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# **DNA methylation variation in lean and obese placenta**

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## Abstract

One in five pregnant women is obese in the UK. Obesity in pregnancy is associated with risks of complications for the mother and child. Mechanisms linking maternal obesity with adverse outcomes for the offspring are not entirely understood though recent studies have suggested epigenetic modifications may be important. As the placenta plays a key role in fetal nutrition, metabolism and protection we hypothesized that there would be DNA methylation changes between lean and obese placenta. DNA methylation array (Infinium HD Assay) was carried out on placentas collected from n=31 obese (BMI>40 kg/m<sup>2</sup>) and n=29 lean (BMI<25 kg/m<sup>2</sup>) women. Three genes miR-411, *HSD17B4* and *FABP1*, with false discovery rate (FDR) adjusted P<0.05, and potentially relevant to maternal obesity, gestational diabetes or fat metabolism after literature review, were selected for validation of DNA methylation by pyrosequencing and measurement of mRNA levels by RT-qPCR. The mean (sd) miR-411 and FABP1 DNA methylation percentage was significantly higher in obese placenta vs lean group (68.9 (13.4)% vs 58.9 (15.8)%, P=0.01 and 89.7(2.76)% vs 85.8(7.16)%, P=0.01, respectively), in accord with the array findings. There were no differences in HSD17B4 DNA methylation between groups. MiR-411 DNA methylation was significantly higher in samples from non-smoking obese vs non-smoking lean (70.42(9.6)% vs 58.63(17.4)%, P=0.02). MiR-411 DNA methylation percentage was highest in placentas from male babies born to obese

mothers. There were no significant differences in mRNA levels of miR-411 between obese and lean groups and there were no correlations between methylation levels and mRNA levels of miR-411 in either obese or lean placentas (miR-411 obese  $r=0.21$ ,  $P=0.32$ ; lean  $r=-0.32$ ,  $P=0.14$ ). MiR-411 mRNA levels were significantly higher in current smokers vs non-smokers and ex-smokers (3.61(3.2) vs 1.3(1.2) vs 1.13(0.7),  $P\leq 0.05$ ) in all placenta samples. Infant BMI was also positively correlated with mRNA levels of miR-411 in lean but not obese group (lean  $r=0.636$ ,  $P=0.003$ ; obese  $r=0.021$ ,  $P=0.931$ ). Maternal age was negatively correlated with miR-411 mRNA levels in obese placenta ( $r=-0.42$ ,  $P=0.05$ ). We were unable to detect FABP1 mRNA in either lean or obese placenta. Hence, we conclude that there are differences of DNA methylation and gene expression between lean and obese placenta, and these differences are also influenced by maternal environment, fetal sex and infant BMI. The explanation for the lack of association of DNA methylation changes and gene expression changes is not known but may be due to the small magnitude of the DNA methylation changes. Further studies are needed to understand the functional outcomes of these DNA methylation changes. Exploration of the regulation pathway, down-stream genes and function of miR-411 is a potential avenue for future work.

## **Lay summary**

One in five pregnant women is obese in the UK. Obesity in pregnancy is known to be harmful for the health of both mother and child. It is still not clear why and how the body weight of mothers could influence the health of their babies. Recent studies have suggested that DNA methylation might be important. DNA methylation is a process which regulates the way genes are turned “on” and “off”. As the placenta plays a key role in fetal nutrition, metabolism and protection, we hypothesized that DNA methylation would be different in the placentas from normal weight compared with obese pregnant women. We measured the DNA methylation in 60 placentas (31 from obese mothers, 29 from normal weight mothers), and identified three genes (miR-411, HSD17B4 and FABP1) for validation in the top list of DNA methylation changes in this panel. Existing literature shows all these three genes are potentially relevant to maternal obesity, diabetes during pregnancy or fat metabolism. After analyzing the gene expression and DNA methylation, we found the DNA methylation changes in miR-411 and FABP1 were higher in the placentas from the obese mothers compared to the normal weight mothers. We could not replicate the findings of differences in HSD17B4 DNA methylation between groups. Intriguingly we found no correlation between DNA methylation changes and gene expression. However, we showed that there were other factors that were associated with changes in either DNA methylation or expression of these genes. For example, mothers who were smoking

during pregnancy had more changes in gene miR-411 expression than those mothers who quit smoking before pregnancy or who had never smoked. We also found that the baby boys who were born from obese mothers had the highest miR-411 DNA methylation changes. Furthermore the changes in miR-411 gene expression were linked to the baby BMI. We conclude that DNA methylation is different in placentas from obese and normal weight mothers. We showed that maternal smoking and baby sex can influence both the DNA methylation and gene expression in placentas. Though we found no links between DNA methylation and gene expression, this might be because of mother behaviours, such as smoking, and baby sex, etc. In future studies we plan to investigate whether these DNA methylation changes influence the health of the baby. In addition, our work has highlighted that miR-411 could be a novel gene to investigate further and understand its role in transmitting the adverse effects of maternal obesity in pregnancy to the baby.



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## Abbreviation

AGEs	advanced glycation end products
AHRR	aryl-hydrocarbon receptor repressor
BMI	body mass index
BCAA	branched-chain amino acid
CXCR2	Chemokine (C-X-C Motif) Receptor 2
DBP	D-bifunctional protein
DOHaD	Developmental Origins of Health and Disease
DMR	differentially methylated regions
DNMTs	DNA methyltransferase
ERTBB	Edinburgh Reproductive Tissue Bio-Bank
EPHB6	Ephrin type-B receptor 6
FDR	false discovery rate
FABP1	fatty acid binding protein 1
GDM	gestational diabetes mellitus
GWG	gestational weight gain
GLUT4	glucose transporter 4
hCG	human chorionic gonadotropin
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HSD17B4	hydroxysteroid 17-beta dehydrogenase 4
INMT	indolethylamine N-methyltransferase
IGF-2	Insulin-like growth factor 2
LGA	large for gestational age
IL-8	interleukin-8
LCFAs	long-chain fatty acid
LINE-1	Long Interspersed Element-1
MMP7	matrix metalloproteinase-7

miR-411	microRNA-411
mtDNA	mitochondrial DNA
TSPO	mitochondrial translocator protein
MCP-1	monocyte chemoattractant protein 1
NAFLD	nonalcoholic fatty liver disease
NFKB1	Nuclear factor NF- kappa -B p105 subunit
OA	oleic acid
PPARs	peroxisome proliferator-activated receptors
PI3K	phosphatidylinositol 3-kinase
PI	Ponderal index in kg/m <sup>3</sup>
pTFR1	placental transferrin receptor
PE	preeclampsia
PPARGC1A	proliferator- activated receptor- $\gamma$ co-activator 1 $\alpha$
ROS	reactive oxygen species
S.D.	standard deviation
SDS_BMI	Standard Deviation Score of birth BMI
SDS_bw	Standard Deviation Score of birth weight
SO	severely obese
TNF $\alpha$	tumor necrosis factor alpha
TBP	TATA-box binding protein
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation
protein zeta	

# **1 Introduction**



## 1.1 Obese pregnant women

The prevalence of obesity, defined as a body mass index (BMI) 30 kg/m<sup>2</sup> or greater, among the general population is increasing worldwide. In the UK, one in five women are obese during pregnancy (Heslehurst *et al.*, 2010). This epidemic has raised concern due to the increasing risk of adverse maternal and fetal health outcomes. Obese pregnant women face a high risk of pregnancy complications, such as hypertension, gestational diabetes and miscarriage (Gaillard *et al.*, 2011; Leng *et al.*, 2015), and their offspring are exposed to increased risk of macrosomia, large for gestational age (LGA) infants and still birth (Ehrenberg, Mercer and Catalano, 2004; Kristensen *et al.*, 2005; Heude *et al.*, 2012). In the Developmental Origins of Health and Disease (DOHaD) hypothesis, the key role of environmental exposures during prenatal and perinatal periods on the adverse children later health and development, including increased risk of cardiovascular and metabolic diseases (Gluckman and Hanson, 2006) is described. The hypothesis has been extended to include environmental exposures such as obesity. Thus maternal overweight and over nutrition have been associated with fetal adiposity, metabolic changes in the offspring including type 2 diabetes (Lake, Power and Cole, 1997; Dabelea *et al.*, 2000) and increased branched-chain amino acid (BCAA), which is positively associated with central adiposity (Perng *et al.*, 2014), as well as increased risk of cardiovascular disease and premature mortality (Reynolds *et al.*, 2013; Eriksson *et al.*, 2014). Fetal programming may also be influenced by environmental stress

factors, such as maternal depression, anxiety, dietary habits/obesity and smoking (Smedts *et al.*, 2009; Joubert *et al.*, 2012), which may have long-lasting health influences on the offspring (Bird, 2002).

## **1.2 Placental function during obese pregnancy**

The placenta is a unique connection between the mother and fetus and has multiple-functions in this relationship. The placenta offers nutrition, transfers oxygen to maintain the growth of fetus by exchange blood from the mother to the fetal side, and it also transports waste and carbon dioxide that come from the fetus. The placenta also plays a key role in the fetal immune system by passing IgG antibodies to the fetus (Simister, 2003). Another important function of the placenta is endocrine. During pregnancy, the placenta releases hormones which are vital for fetal growth, such as the human chorionic gonadotropin (hCG) hormone; Progesterone is important for embryo implantation and offers protection from spontaneous abortion (Hassan *et al.*, 2011); Estrogen is also crucial for fetal growth by mediating the development of breast, playing an important role in breastfeeding and fetal health (Briskin and O'Malley, 2010).

The placenta is such an important organ during normal implantation: the cytotrophoblast cells invade the maternal surface, and transform maternal smooth muscle and decidual vascular endothelial cells to form endovascular cytotrophoblast,

while the endovascular cytotrophoblast cells form high-caliber capacitance vessels allow maternal transferring oxygen and nutrients to fetus through placenta (Damsky, Fitzgerald and Fisher, 1992). These transferring pathways are influenced by the thickness of the placenta membrane and the number of transporters in the trophoblast membrane. During normal pregnancy, both expansion of the placenta membrane and reduction in thickness of the interhaemal membrane were observed in human and in a mouse model (Coan, Ferguson-Smith and Burton, 2004; Mayhew, 2008). Apart from the morphology changes during normal pregnancy, the changes of levels of hormones in the maternal and fetal circulations were also demonstrated in early studies. The syncytiotrophoblast secretes a variety of hormones and growth factors to help maintain the normal function of placenta and the communication between maternal and fetal during pregnancy. For example, estrone is secreted from maternal ovaries or adrenal in early pregnancy. Placenta later starts to secrete estrone which is sourced from maternal circulation and the concentration of estrone is continually increased until the late stage of gestation (Tulchinsky and Hobel, 1973; Lindberg, Nilsson and Johansson, 1974).

Therefore, the placenta is crucial to normal pregnancy and fetal development. Dysfunction of placenta with different pregnant complications (diabetes, preeclampsia and obesity, etc.) and the association with fetal outcome has been

discussed in many studies (Baschat and Hecher, 2004; La Torre *et al.*, 2006; Van den Bergh *et al.*, 2008).

In this study, we mainly focus on the changes that happen in obese placenta. Dysfunction of placenta in women with obesity and the association with fetal development has been discussed in a number of studies. The placenta is a major source of cytokines during pregnancy (Bowen *et al.*, 2002), tumor necrosis factor alpha (TNF $\alpha$ ) is secreted to both mother and fetus. During placental inflammatory disorders, such as under obese pregnant conditions (Challier *et al.*, 2008; Du *et al.*, 2010), inflammation was suggested to be a reason contributing to adverse fetal development, such as preterm birth and neonatal brain damage, with associated fetal and neonatal morbidity (Redline, 2004). Moreover, some inflammatory mediators, mRNA levels of inflammation relevant cytokines including interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) and Chemokine (C-X-C Motif) Receptor 2 (CXCR2) were higher in the placenta of obese pregnant women than lean pregnant women, and higher IL-6 levels were also found in the maternal circulation with obesity compared with lean women (Roberts *et al.*, 2011), suggesting that obesity is associated with increased inflammation during pregnancy. In another study, an increase in macrophages and pro-inflammatory mediators were reported in the placenta of overweight pregnant women compared to normal weight women

(Challier *et al.*, 2008). Apart from obesity induced placental inflammation, morphological changes in placenta from obese women were also described in a few studies. The activity of mitochondria was lower in obese placenta as well as the decreased mitochondrial DNA (mtDNA content), while the mitochondrial oxidative stress, which was measured by mitochondrial hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels, was increased in the obese placenta compared with non-obese placenta (Hastie and Lappas, 2014). The authors suggested that these changes in mitochondria will increase the level of reactive oxygen species (ROS). The production of ROS is involved in embryo implantation and development, so any changes in ROS could lead to altered fetal growth (Lappas, 2014). A recent study which recruited 234 pregnant women, demonstrated the transporters of placental mitochondrial cholesterol and the mitochondrial translocator protein (TSPO) were significantly decreased in pregnant women with obesity. The study demonstrated that cholesterol plays a main role in estrogen and progesterone synthesis in placenta and TSPO is the cholesterol binding protein; thus their study suggested the changes of mitochondrial cholesterol will largely influence placental function and pregnancy losses (Lassance *et al.*, 2016). In a study from Hayward *et al.*, in which chorionic plate arteries (CPAs) were pre-constricted followed by sodium nitroprusside (SNP, a vasodilator) treatment, the vasodilation in CPAs was decreased in overweight pregnant women. This would potentially influence the transportation of oxygen and nutrition between mother and

fetus (Hayward *et al.*, 2013). Another study reported that placental villous proliferation and apoptosis had a positive association with maternal weight. Changes in placenta size and integrity of the placental barrier will potentially influence the transfer of nutrition and oxygen between maternal and fetus, and those changes could influence pregnancy outcomes (Higgins *et al.*, 2013). In terms of the influence of placental dysfunction on nutrition transport, one study demonstrated obese pregnant women had lower circulating levels of iron but the placental transferrin receptor (*pTFR1*) expression in placenta was higher than lean pregnant women, which indicates that placenta could protect itself by adaption to the potential adverse fetal environment which is associated with obesity (Garcia-Valdes *et al.*, 2015). In another study, a decreased expression of insulin-regulated glucose transporter 4 (*GLUT4*) mRNA in obese placenta was described. They found the dysregulation of *GLUT4* could result in increased levels of circulating free glucose, which implies obesity during pregnancy may have impact on the insulin signaling pathway. The influence of *GLUT4* and phosphatidylinositol 3-kinase (PI3K) on glucose taken to cell membrane could induce gestational diabetes (Colomiere *et al.*, 2009). There are some studies suggesting that the placental responses to maternal weight are fetal sex dependent. To be more specific, Brass *et al.* (2013) carried out a study using human placentas and showed that female offspring could uptake a higher placental monounsaturated fat, oleic acid (OA), which was studied as a biomarker of

cardiovascular disease (Schwingshackl, Strasser and Hoffmann, 2011). In obese mothers male offspring showed lower placental oleic acid uptake, which suggested that the male fetus is more vulnerable than the female fetus under the environment of maternal obesity (Brass *et al.*, 2013). From the studies mentioned above, we could conclude that the placenta might play a protective role on fetal growth during pregnancy from maternal different conditions and the function of placenta could be influenced by pregnancy environment and different fetal status too.

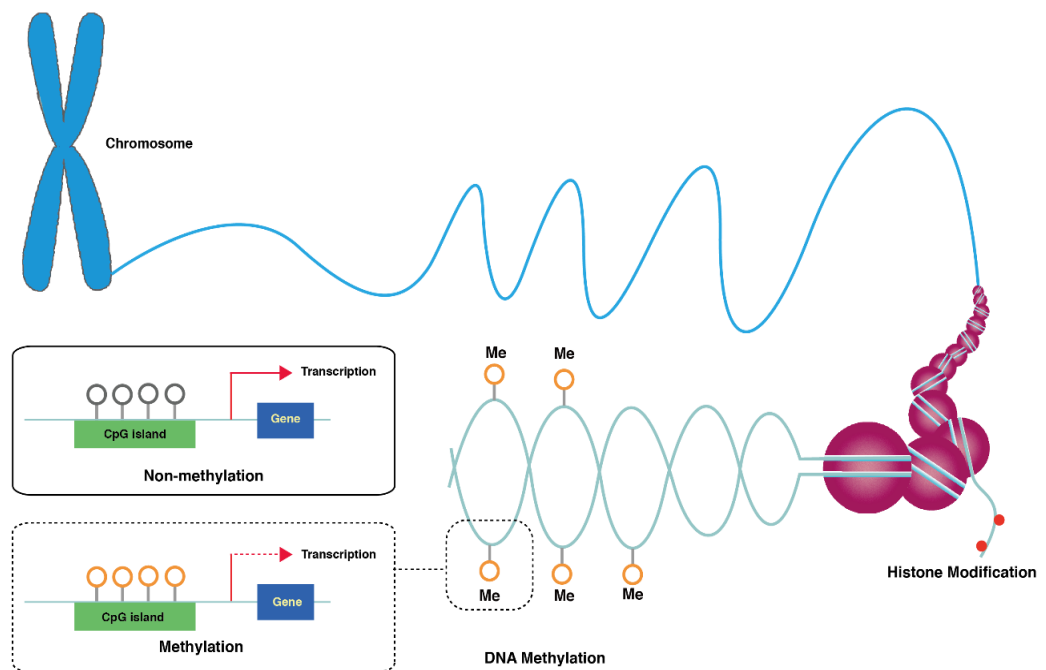
### **1.3 Epigenetic modification and DNA methylation**

Epigenetic modification is defined as a key pathway to regulate gene expression stabilization. The definition of “epigenetic” was first mentioned by Waddington (Waddington, 1956), and has been defined as ‘the inheritable changes in gene expression due to the influence of environment factors and without changes of DNA sequence’ (Jablonka and Lamb, 2006).

The mechanisms of epigenetic modification include DNA methylation, histone modification and non-coding RNAs (Figure 1). DNA methylation is one of the common epigenetic mechanisms and has been most studied in the context of fetal developmental programming (Waterland and Jirtle, 2004; Gentilini *et al.*, 2013). The process of DNA methylation is the addition of a methyl group to the 5’carbon of cytosines, mainly in the context of cytosine-guanine dinucleotides, a process which is catalyzed by the DNA methyltransferases (DNMTs) and this largely results in the

repression of gene transcription. Groups of unmethylated CpGs are named as ‘CpG islands’, which are mainly located in promoters and at the 5’ end of genes. Methylation in the ‘CpG islands’ is highly relevant to gene inactivity (Godfrey, Costello and Lillycrop, 2015). DNA methylation is observed in most mammals, including human, mouse and sheep (Santos *et al.*, 2002; Beaujean *et al.*, 2004; Joubert *et al.*, 2012). DNA methylation also occurs in specific tissues, for example, embryonic stem cells, germ cells and trophoblast stem cells. Those cells all have their own unique DNA methylation patterns (Ohgane, Yagi and Shiota, 2008). Moreover, DNA methylation is associated with imprinted gene and X chromosome inactivation (Jones and Takai, 2001). Thus, analyzing DNA methylation is an important way to investigate epigenetic modification and programming.





**Figure 1.** The process of epigenetic modification. DNA methylation: methylated sites on the gene could influence the activity the gene. Histone modification: the epigenetic factors binding on histone “tails” could change the activity of the DNA wrapping on the histone.

### **1.3.1 Epigenetic modifications in obese pregnancy**

A number of studies have started to investigate whether epigenetic modifications may occur during obese pregnancy though whether these are linked to adverse offspring outcomes is still unclear. Different types of samples were used in the studies comparing DNA methylation expressions between obese and lean pregnant women. A summary of the available literature about DNA methylation between lean and obese pregnant women is shown in Table 1.

In total, five studies investigated differences in individual genes' methylation according to maternal obesity by using umbilical cord blood. Another five studies analyzed global gene methylation by using umbilical cord blood and one study using maternal visceral and abdominal fat tissues. The variables of maternal weight, BMI, Gestational weight gain (GWG), maternal glucose and birth weight have all been analyzed in these studies to test whether they are associated with DNA methylation differences in placenta and offspring.

Umbilical cord blood is one of the most common tissue samples used in DNA methylation studies, as cord blood can be immediately and easily collected after birth. Promoter methylation of the proliferator- activated receptor- $\gamma$  co-activator 1 $\alpha$  gene (*PPARGC1A*) which is relevant to gluconeogenesis (a process to generate glucose from non-carbohydrate carbon substrates), was found positively corresponding with maternal BMI in a study with 88 healthy pregnant women (Gemma *et al.*, 2009).

However, in another study, which recruited pregnant women with GDM (gestational diabetes mellitus), *PPARGC1A* promoter methylation in cord blood was found negatively associated with maternal glucose concentration and obesity (Xie *et al.*, 2015). Insulin-like growth factor 2 (*IGF-2*) DNA methylation was reported as decreased in obese cord blood, whereas aryl-hydrocarbon receptor repressor (*AHRR*) was increased in obese pregnant women (Hoyo *et al.*, 2012; Burris *et al.*, 2015). No association of LINE-1 (Long Interspersed Element-1) methylation was found with BMI in both cord blood and placenta, but the methylation of LINE-1 was lower in low birth weight/ high birth weight fetus (Michels, Harris and Barault, 2011). Other studies have demonstrated the changes of global DNA methylation in obese pregnant women compared with lean pregnant women. GWG showed no association with global DNA methylation while maternal underweight was positively linked with fetal DNA methylation and there was no differences of CpG sites methylation in between overweight and normal pregnant women (Sharp *et al.*, 2015). Greater GWG was also described with increasing DNA methylation in CpG sites in gene matrix metalloproteinase-7 (*MMP7*) and Nuclear factor NF- kappa -B p105 subunit (*NFKB1*) etc. (Morales *et al.*, 2014). A similar study carried out by Herbstman *et al.* using umbilical cord blood, also supports the view that BMI was negatively associated with global DNA methylation (Herbstman *et al.*, 2013). By contrast, another study demonstrated increasing BMI was associated with high levels of fetal DNA

methylation as well as birth weight (Liu *et al.*, 2014). *IGF2* differentially methylated regions (DMR) – such as H19 DMR, was found increasing with maternal weight (Soubry *et al.*, 2013).

Visceral and abdominal fat tissues were also used in one study to analyze the differences of DNA methylation between lean and obese pregnant women. In one study, DNA methylation of indolethylamine N-methyltransferase (*INMT*) and Ephrin type-B receptor 6 (*EPHB6*) were lower in obese group compared with lean pregnant women (Bashiri *et al.*, 2014). There are three papers demonstrating DNA methylation in the placenta. One study demonstrated a positive association of *PPARGC1A* DNA methylation levels in placenta with maternal glucose level (Côté *et al.*, 2016), which is consistent with the study performed by Xie *et al.* (2015). Whether there was a correlation of DNA methylation with maternal BMI and infant birth weight was not tested in either study. In another study, there was no association between placenta DNA methylation and maternal BMI (Michels, Harris and Barault, 2011). The reason for those differences in maternal BMI with *PPARGC1A* DNA methylation might due to different samples tested in each study (umbilical cord vs placenta), suggesting the possibility that DNA methylation can be varied from different tissues. Another reason is different demographic characteristic of the women from which the samples were collected and included in each study (e.g. with/without women with GDM).

Gene symbol	Design	Country	mechanism	Tissue	Study sample	Methodology	Findings	References
<i>PPARGC1A</i> , <i>PPARG</i>	cross-sectional study	China	DNA methylation	Unbilical cord blood; placenta	58 Healthy pregnant women	1. Bisulfite treatment: using an EZ DNA Methylation Gold kit (Zymo Research) to convert unmethylated cytosines (C) into thymine (T) 2. Pyrosequencing; four primer assays; PyroMark assay design software and PCR reagents	The maternal gestational glucose level was positively correlated with placental DNA methylation, and negatively correlated with cord blood DNA methylation of the <i>PPARGC1A</i> promoter in a CpG site-specific manner. In the GDM group alone, the placental CpG site-specific methylation of the <i>PPARGC1A</i> promoter strongly correlated with gestational 2-h post-OGTT glycaemia.	(Xie et al., 2015)
<i>PPARGC1A</i> , <i>PPARG</i>	cross-sectional study /		DNA methylation	Unbilical cord blood	88 Healthy pregnant women and their babies	1. Bisulfite treatment 2. MethPrimer program; SYBR; two pairs primers (Methylation/unmethylation)	PARG promoter was almost 100% methylated in SGA&LGA samples. In univariate analysis, there was no association among characteristics of the newborn and gene promoter methylation. None of the maternal features were related with the status of promoter methylation, except for a positive correlation between maternal BMI and <i>PPARGC1A</i> promoter methylation in umbilical cord (Pearson correlation coefficient $r = 0.41$ , $P = 0.0007$ ).	Gemma et al. (2009)
gestational weight gain	general population pregnancy cohort study	UK	DNA methylation	cord or peripheral blood	a subset of 1018 mother-offspring pairs	1. Bisulfite treatment 2. DNA methylation in cord or peripheral blood was measured using the Illumina InfiniumVR HumanMethylation 450K BeadChip assay at the University of Bristol ( <a href="http://ije.oxfordjournals.org/content/suppl/2015/03/26/dy042.DCI/Supplementary_text_figures_and_tables.pdf">http://ije.oxfordjournals.org/content/suppl/2015/03/26/dy042.DCI/Supplementary_text_figures_and_tables.pdf</a> )	1. maternal underweight has a larger effect on the fetal epigenome than maternal obesity does. At most sites, maternal body mass index outside the normal range was associated with higher offspring methylation. The effect of maternal obesity was stronger than the effect of paternal obesity, supporting an underlying intrauterine mechanism. 2. There were no consistent associations of gestational weight gain with DNA methylation in this study.	(Sharp et al., 2015)
IGF-2	the Newborn Epigenetics Study (NEST), a multiethnic cohort study	North Carolina	DNA methylation	Unbilical cord blood	300 pregnant women	1. Bisulfite treatment 2. Pyromark Q96 MD Pyrosequencing instrument	Lower IGF2 DMR methylation was associated with elevated plasma IGF2 protein concentrations, and this association was stronger in infants born to obese women (pre-pregnancy)	(Hoyo et al., 2012)
<i>MMP7</i> , <i>KNK4</i> , <i>TRPM5</i> and <i>NFKB1</i>	longitudinal Study of Parents and Children (ALSPAC),	UK	DNA methylation	Off-spring cord blood	88 mother-offspring pairs	1. Bisulfite treatment 2. Illumina Golden Gate assay kit 3. Validation study : a PyroMark MD Pyrosequencing System (Qiagen) was used for the validation of the top associated locus ( <i>MMP7_E59</i> ).	Greater GWG in early pregnancy was associated with increased methylation at CpG sites at <i>MMP7</i> , <i>KNK4</i> , <i>TRPM5</i> and <i>NFKB1</i> genes in offspring cord blood DNA. The specific association of GWG in early pregnancy with the top associated CpG site at <i>MMP7</i> was not validated using Pyrosequencing and it did not replicate	(Morales et al., 2014)
<i>INMT</i> , <i>EPHB6</i>	Case control study	Israel	DNA methylation	visceral and abdominal fat tissues	4 each in obese and lean group	The Affymetrix Human Exon 1.0 ST Array (Lockstone 2011), a validated high-density gene expression microarray platform with close to 5.5 million probe sets, was used in this study. cDNA was generated with GeneChip WT cDNA Synthesis and Application Kit (Affymetrix)	1. INMT over expressed in visceral fat of normal weight pregnant women, and appear to be up-regulated in visceral fat of obese women potentially implicating deregulation of cell-to-cell signaling and cellular growth in visceral fat of obese individuals in pregnancy. 2. <i>EPHB6</i> , the significant alterations appear to be attributable to the decrease in expression in the subcutaneous adipose of the obese women as compared to subcutaneous fat of normal weight group ( $p < 0.05$ )	(Bashiri et al., 2014)

**Table 1.** Current study about DNA methylation in lean and obese pregnant women. Classified by study design, country, mechanism, tissue, study sample, methodology, findings and references.

<i>LINE-1</i>	Epigenetic Birth Cohort	USA	DNA methylation	Cord blood and placenta	319 mother-child pairs	1. Bisulphite treatment 2. Pyrosequencing : a Pyromark Q24 Pyrosequencing instrument	They did not find important associations between maternal pre-pregnancy BMI or gestational weight gain and global methylation of the cord blood or fetal placental tissue, but they found lower levels of LINE-1 methylation in cord blood among newborns with low and high birthweight as well as among prematurely born infants than normal birth.	(Michels et al., 2011)
<i>AHRR</i>	prospective birth cohort study, Programming Research in Obesity Growth Environment and Social Stress (PROGRESS)	Mexico	DNA methylation	Cord blood	512 mother-child pairs	1. Bisulphite treatment 2. MethPrimer online tool44 ( <a href="http://www.urogene.org/cgi-bin/methprimer/meth-primer.cgi">http://www.urogene.org/cgi-bin/methprimer/meth-primer.cgi</a> ) to design PCR primers for use with pyrosequencing and bisulfite-converted DNA. Pyromark Q96 MD Pyrosequencing instrument	AHRR DNA methylation was 2.1% higher in offspring of obese vs. normal weight mothers and 3.1% higher in preterm vs. term infants, representing a third and a half standard deviation differences in methylation, respectively.	(Burris et al., 2015)
pre-pregnancy BMI	The Northern Manhattan Mothers and Newborns Study of the Columbia Center for Children's Environmental Health (CCCEH); a longitudinal cohort study	African American and Dominican women	DNA methylation	Cord blood; blood	165 children	MethylampTM Global DNA Methylation Quantification Kit (Epigentek Group Inc, NY)---quantifies the methylated fraction of DNA using an ELISA-like reaction	1. DNA methylation was significantly higher in blood at age 3-years than in cord blood; 2. women who were obese prior to pregnancy had the lowest DNA methylation, thus pre-pregnancy BMI was negatively predictive of both cord and three-year DNA methylation	Herbstman et al., 2013
ZCCHC10, WNT16, ACPL2, PLAC1	the Boston Birth Cohort (BBC)	African American and Haitian	DNA methylation	cord blood	309 children (questionnaire: mother information)	the Illumina HumanMethylation27BeadChip	1. mothers who had a higher BMI appeared to have heavier babies; 2. The methylation levels of 20 CpG sites were associated with maternal BMI at a significance level of P-value <0-4 in the overall sample, and boys and girls, separately. (cancer, immune, cardiovascular)--- pre-pregnancy maternal BMI might play a more critical role in infection and inflammation for boys; while infectious and inflammatory response pathways and lipid metabolism, cardiovascular diseases, cancers, and endocrine system disorders are indicated for girls.	Liu et al., 2014
IGF2 (differentially methylated regions)DMR, H19 DMR	the Newborn Epigenetics Study (NEST)	African American, Caucasian, or other not specified	DNA methylation	cord blood	79 newborns (questionnaire: mother/father information)	Pyromark Q96 MD pyrosequencing instrument (Qiagen)	1. significantly lower methylation at the IGF2 DMR associated with paternal obesity, and significantly higher methylation at the H19 DMR when the mother was obese. 2. Education was not associated with obesity in either of the parents, and race was only associated with obesity of the mother.	Soubry et al., 2013

Therefore, epigenetic modification is a potential mechanism to investigate the association between maternal obesity and development of disease in childhood and adult life. In this study, we used placenta as a target tissue to analyze whether there are differences in DNA methylation between lean and obese women, as few studies in the literature have focused on the analysis of placenta tissues in lean and obese groups and only a few studies have big sample sizes.

#### **1.4 Aim and hypothesis**

The aim of this study was to investigate whether there are differences in DNA methylation in obese and lean placenta by using a DNA methylation array and by validating key genes found to be different between lean and obese by pyrosequencing. The hypothesis was that there would be higher DNA methylation in obese placenta than lean placenta, and that gene expression would be lower in obese than lean groups.

## **2 Materials and Methods**



## 2.1 Samples and recruitment

Placenta samples for DNA extraction were from a prospective cohort study of very severely obese (SO) pregnant women in Edinburgh, Scotland, UK (Mina *et al.*, 2015a). Briefly, pregnant women were defined as lean ( $\text{BMI} \leq 25 \text{ kg/m}^2$ ) and SO ( $\text{BMI} \geq 40 \text{ kg/m}^2$ ) women at antenatal booking. Lean pregnant women with singleton pregnancies were recruited from community antenatal clinics and SO pregnant women with singleton pregnancies were recruited from a specialist antenatal metabolic clinic (Mina *et al.*, 2015a). Ethical approval and written informed consent were obtained (references 08/S1101/39 and 13/ES/0126). In a previous study using placental samples from this cohort it has been demonstrated that maternal distress was associated with changes in placental mRNA genes regulating fetal glucocorticoid exposure, with sex-specific differences such that female fetuses were more vulnerable to glucocorticoid exposure (Mina *et al.*, 2015b).

A total number of 93 placental samples (43 lean and 50 obese) were selected from the Edinburgh Reproductive Tissue Bio-Bank (ERTBB). Women with gestational diabetes, treated with antenatal steroids during pregnancy and preterm birth (< 36 weeks) were excluded. From these, we analyzed data from 60 placental samples (exclude missing data in maternal weight/BMI and bad quality samples from RNA extraction) with lean (n=29) and obese (n=30) pregnant women samples. Five samples were excluded from the sample pool as we found the records of those

samples in ERTBB, such as birth weight, were not matched with the record from the hospital.

## **2.2 Placenta collection and DNA extraction from placenta tissues**

Human placenta samples were collected from ERTBB as detailed in Mina et al (2015b). Placental tissues were collected from the center side of the placenta and were stored in RNAlater solution (Qiagen, Manchester, UK) at 4 °C for 24 h and subsequently frozen at –80 °C.

We applied the QIAamp® DNA Mini Kit (Qiagen, Crawley, West Sussex, UK) protocol for DNA isolation from fibrous tissues. Approximately 30 mg placenta tissue was cut and added to a 1.5 ml microcentrifuge tube with 180 µl lysis buffer ATL and 20 µl proteinase K mixture. The tube was next incubated in a rotating air incubator at 56 °C overnight to ensure complete tissue lysis. On the following day RNase A (4 µl, 100 mg/ml; Qiagen, Crawley, West Sussex, UK) and buffer AL (175 µl) were added to each sample for homogenized and the mixtures were incubated at 70 °C for 10 minutes. Samples were subsequently transferred to another set of clear tubes and mixed with 100% ethanol (200 µl) followed by a quick spin at 6000 xg for 2 minutes. Samples were then transferred to the QIAamp Mini spin column, and washed with buffer AW1 and AW2 as per the manufacturer's handbook and the final DNA samples were eluted in 150 µl of elution buffer AE.

## **2.3 DNA qualification and verification of integrity**

The concentration and quality of isolated DNA were evaluated using the Nanodrop (Thermo-Scientific, Loughborough, UK) and the reading of 260/280 and 260/230 ratio. A good quality of DNA is generally defined by the reading of 260/280 ratio above 1.8 and the ratio of 260/230 for good nucleic acid is around 2.0-2.2 and usually higher than the corresponding 260/280 value. The integrity of the extracted DNA samples was determined by running a 1% agarose gel mixture with diluted 1X TBE from 10X TBE (Tris base 121.1 g, Boric acid 61.8 g, EDTA 7.4 g) in 0.5X TBE buffer for 90 minutes at 70 voltages. The gel was visualized under UV light in the UV Transilluminator (Uvitec, Cambridge, UK) following a gel electrophoresis.

## **2.4 Epigenetic database built (gene and promoter)**

### **2.4.1 Candidate gene selection**

DNA methylation array was conducted using the Illumina Methylation 450k beadchip at the Wellcome Trust Clinical Research Facility generating data on 485,000 CpG sites. Briefly, after bisulphite treatment, bisulphite-converted DNA was processed by using the Infinium HD Assay for methylation. The images for each arrays and genotypes were recorded automatically using GenomeStudio Analysis software (version 2011.1).

The whole process of DNA methylation analysis can be separated in four steps and

the data were generated by using the RnBeads tool v 0.99.17 (Sparrow *et al.*, 2016). First step was data loading followed by pre-processing. The next stage was normalisation using the beta mixture quantile dilation method (Teschendorff *et al.*, 2013). Finally, differential methylation between lean and obese pregnant women were assessed in gene bodies and promoters (by Dr Jon Manning). RnBeads (v77 version) was used to analyse the different DNA methylation data and the regions of promoters are from 1.5kb upstream to 0.5kb downstream of the 5'-end (transcription start site). The false discovery rate (FDR) was used to select significant methylated genes/promoters.

In total 288 genes were chosen from 27411 candidate genes identified as those that had a significant DNA methylation differences in the gene body after FDR adjustment between lean and obese ( $P < 0.05$ ). By excluding genes' name as unknown ( $n=157$ ) and the genes with a name but no information in PubMed/Web of Science/Google Scholar ( $n=60$ ), the total number of genes which could have relevant information (key words: obesity/BMI/overweight, pregnancy, human and placenta) in PubMed gene database were 71. Out of these 71 genes, 57 genes were excluded as they were not relevant to obesity or pregnancy, such as basic immune biology ( $n=7$ ); cell interaction ( $n=23$ ); hair ( $n=2$ ); muscle calcium ( $n=1$ ); sperm ( $n=5$ ); gene location ( $n=2$ ) etc. The final potential candidate genes were 14 in total (Figure 2).

#### 2.4.2 Candidate promoter selection

The total number of candidate differentially methylated promoters between obese and lean ( $P < 0.05$ ) were 362. By excluding the promoters with no information in PubMed/Web or Science/ Google Scholar ( $n=180$ ), out of 180 there were 21 promoters each have a 'sibling' promoter to share a same name but different positions. The total number of promoters which could be found with key words 'pregnancy' and 'obesity' in PubMed gene database were 182. Out of these 182 promoters, 19 promoters shared the same name and 142 promoters were excluded as they were not relevant to obese and pregnancy, such as basic immune biology ( $n=17$ ); cell interaction ( $n=43$ ); hair ( $n=6$ ); muscle calcium ( $n=3$ ); gene basic information ( $n=35$ ) etc. The final potential candidate promoters were 40 in total, including 19 promoters with the same name (Figure 3).

Out of those candidate genes and promoters, three genes microRNA-411 (miR-411), fatty acid binding protein 1 (*FABP1*) and hydroxysteroid 17-beta dehydrogenase 4 (*HSD17B4*) were chosen as our final target genes for further investigation. MiR-411 has been found to be significantly down regulated in an experimental model of a diabetogenic-like environment (250 mg/dl glucose and 200  $\mu\text{g}/\mu\text{l}$  advanced glycation end products (AGEs) with human serum albumin) using human umbilical vein cord endothelial cells (Zitman-Gal *et al.*, 2014). However, Zitman-Gal's study is limited as this was an experimental study mimicking the diabetic environment rather than

using samples from women with gestational diabetes. In another study using human placenta samples (mild preeclampsia (n=8), severe preeclampsia (n=15) and normal pregnant women (n=11)), a distinctive down-regulation of miR-411 (P=0.437 fold change) was demonstrated under preeclampsia (PE) conditions. Mild PE was defined as maternal systolic blood pressure of  $\geq 140$  mm Hg and/or diastolic blood pressure of  $\geq 90$  mm Hg on 2 occasions separated by 6 hours and significant proteinuria ( $\geq 300$  mg of protein in a 24-hour urine specimen or  $\geq 1+$  by dipstick) after 20 weeks of gestation. Severe PE was defined as either severe hypertension (systolic blood pressure of  $\geq 160$  mm Hg and/or diastolic blood pressure of  $\geq 110$  mm Hg on at least 2 occasions 6 hours apart) plus mild proteinuria or mild hypertension plus severe proteinuria  $>2$  g/24 hr or  $>2+$  by dipstick). However, this study is limited by the small number of samples in each group (Zhu *et al.*, 2009).

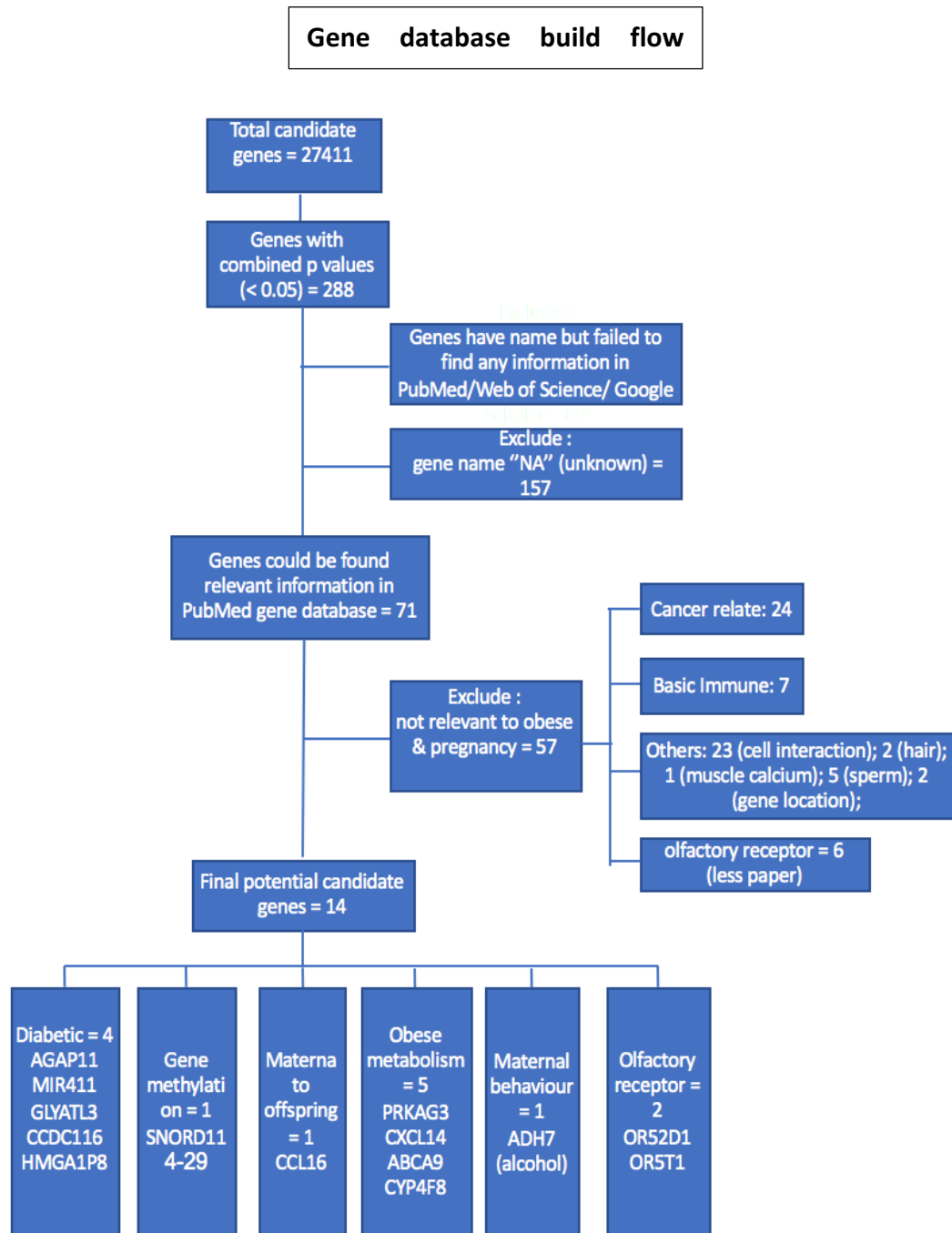
FABP1, also known as liver FABP has been reported to bind or up-take long-chain fatty acid (LCFAs) in the cytosol during LCFA  $\beta$ -oxidation. LCFA  $\beta$ -oxidation is a long process whereby fatty acids are broken down to produce energy. As LCFA is an important mediator of dietary fat uptake, the authors suggested that FABP1 has a potential role in prevention of diet-induced obesity (Atshaves *et al.*, 2010). FABP1 was also suggested to play a vital role in hepatic lipid metabolism. In a study using a mouse model of nonalcoholic fatty liver disease (NAFLD) and human hepatocytes

without steatosis, a significant decrease of *FABP1* gene expression was demonstrated in NAFLD (Guzmán *et al.*, 2013). Rat recombinant L-FABP protein was used in a study which suggested FABP1 could influence glucose homeostasis by binding with glucose (high affinity for glucose,  $K_d=103.1\pm18.6$  nM). The high concentration (20-30 mM) of glucose increased the interaction of FABP1 with peroxisome proliferator-activated receptors (PPARs) and it was suggested that this could consequently affect fat metabolism. A limitation is that this is an animal study (Hostetler *et al.*, 2010). However, *FABP1* deficient mice have been used in many studies aiming to understand the function *FABP1*. A few studies have shown an age-dependent weight gain ( $P<0.05$ , 4 g in male and female at age of 9 months) in both male and female *FABP1* deficient mice. It was also shown that high fat diets could exacerbate ( $P<0.003$ , 1.8 fold change) the body weight in *FABP1* knock-out female mice more than wild type female mice (Martin *et al.*, 2006; Martin *et al.*, 2009; Atshaves *et al.*, 2010).

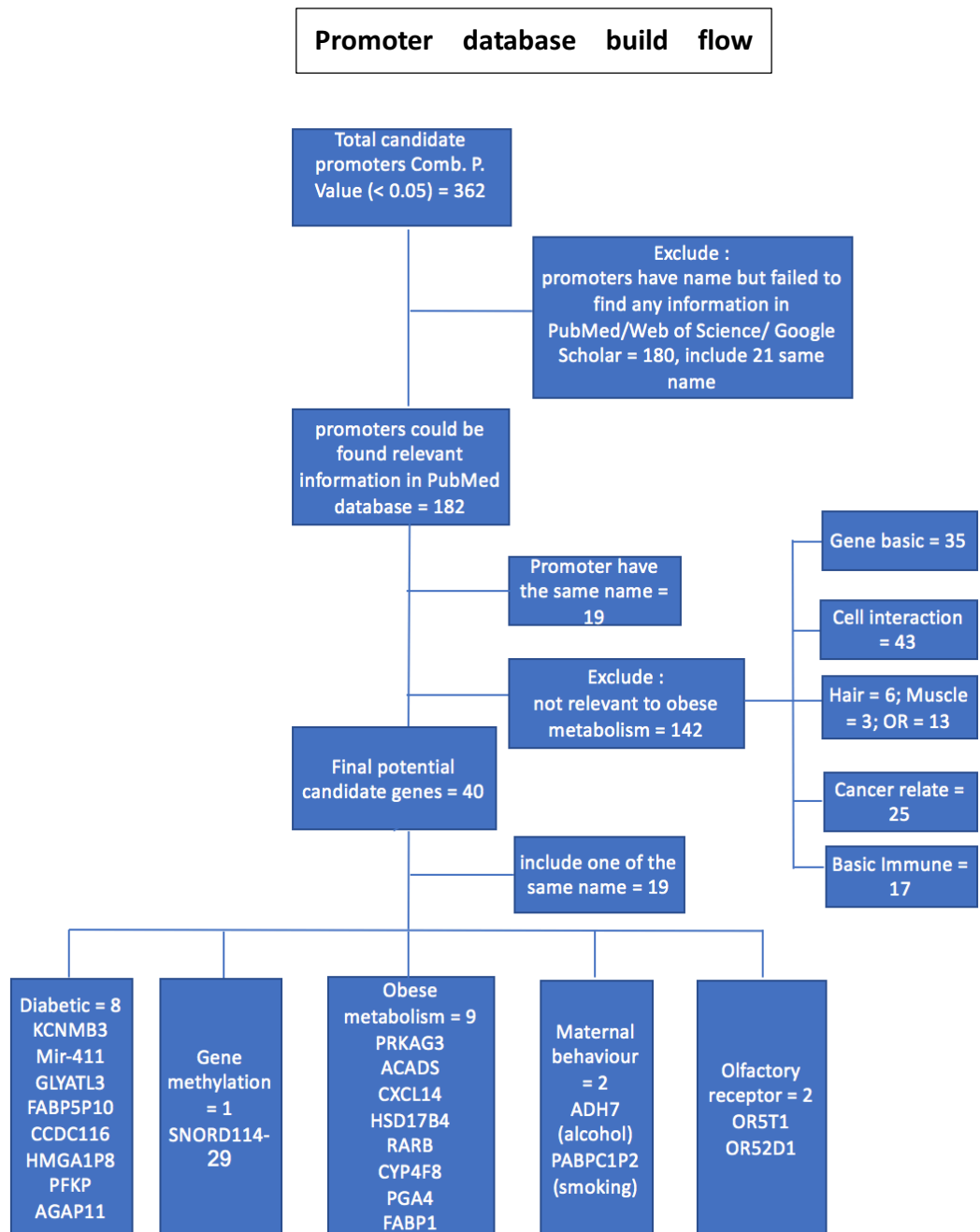
Mutation of *HSD17B4* was found in Zellweger syndrome, a disorder of peroxisomal fatty acid  $\beta$ -oxidation, which results in D-bifunctional protein (DBP) deficiency, and with a phenotype of hypotonia and seizures in early life (Suzuki *et al.*, 1997; van Grunsven *et al.*, 1998; de Launoit and Adamski, 1999). In a recent study, different types of DBP deficiency and the link with *HSD17B4* gene was analyzed. In a single

case report of a newborn with severe hypotonia and craniofacial dysmorphism, a N457Y mutation in the *HSD17B4* gene was reported (Nascimento *et al.*, 2015). High fat diet among female mice was demonstrated to induce gene expression of *Hsd17b4* in mice liver and help with increasing peroxisomal  $\beta$  -oxidation enzymes (Kozawa *et al.*, 2011). Mulberry leaf was described involved in relieving dyslipidemia by upregulation the transport of long fatty acids in previous study (Doi, Kojima and Fujimoto, 2000). In a study using male Wistar rats (outbred albino rat, 5 weeks old, n=30), gene expression of *Hsd17b4* in rats' liver was increased in the group fed with mulberry leaf with high-fat diet, which indicates *Hsd17b4* could also be involved in upregulation of the fatty acid oxidation pathway (Kobayashi *et al.*, 2010).





**Figure 2.** Identification of genes for validation. Total candidate genes were generated from 60 placenta samples. Selection of final candidate genes is basically based on the relevance of the gene to pregnancy, maternal disease, epigenetic changes and fat metabolism.



**Figure 3.** Identification of promoters for validation. Total candidate genes were also generated from 60 placenta samples. Selection of potential promoters is basically based on the relevance of the gene to pregnancy, maternal disease, epigenetic changes and fat metabolism. The final candidate promoters were validated with the final genes we choose.

## **2.5 DNA bisulfite conversion**

EZ DNA Methylation<sup>TM</sup> Kit was used for bisulfite conversion of DNA. Briefly, DNA samples (500 ng) were mixed with M-Dilution Buffer (5 µl) and water to 50 µl following by 15 minutes incubation at 37 °C. CT Conversion Reagent (100 µl) was added to the mixture for 16 hours incubation at 50 °C for 16 hours. M-Binding Buffer (400 µl) and samples were loaded into Zymo-Spin<sup>TM</sup> IC Colimn with collection tube. After four wash steps (according to the manufacturer's handbook), M-Elution Buffer (10 µl) was added to elute the DNA. Followed with 30 µl RN-ase free water dilution for later PCR work. Samples were stored at -20 °C for further use.

## **2.6 Primer validation**

Nine different assays: miR-411(n=2), HSD17B4 (n=3) and FABP1 (n=3), were tested to identify the best primers and temperatures to work with in the subsequent PCR. PCR primers were designed using PyroMark Assay Design Software 2.0 (Qiagen; <https://www.qiagen.com>). In detail, master mix (5µl), primer mix (forward and reverse primers, 0.4 µl), diluted converted test DNA (0.5 µl) and RN-ase free water were mixed to 10 µl. PCR was carried out for 45 cycles at four different temperatures 50°C, 53°C, 56°C and 60°C, in which 50 °C was set according to the manufacturer's handbook with recommended temperature for assays to work in PCR and adjusted in following step by different gradations (53°C, 56°C and 60°C) to find out the best temperature for PCR to work. Water control was used as negative control. Agarose

gel (1%) was ran to test the quality of different assays with PCR samples (10 µl) and 6 X loading dye (2 µl).

## **2.7 Pyrosequencing**

Total volume for PCR were increased to 30 µl, with master mix (15 µl), primer mix (forward and reverse primers, 1.2 µl), diluted converted test DNA (2 µl) and RN-ase free water (11.8 µl) by using the AmpliTaq Gold 360 kit (Applied Biosystems, Warrington, UK). Only 5 µl PCR samples were used for gel image, the rest samples (25 µl) were prepared for pyrosequencing. Pyrosequencing was performed according to the manufacturer's instructions by using PyroMark Gold Q24 reagents on a PyroMark Q24 Pyrosequencer (Qiagen). Data were analysed using PyroMark Q24 1.0.10 software (Qiagen). Details of assays used are shown in Table 2.

Assay name		Primer Sequence
<i>HSD17B4</i>	Pyrosequencing primer	AAAGAAGAGAAAGTTTATTAA
	Forward primer	TTTAGGTTTTTATAGTGGGAAGGTATT
	Reverse primer	Biotin-AAATCCAAAAATATCACCCCTCCATAA
	Sequence to analyze	ATTATAATYGGAAAGTAGAAATTATTTTATTGGA
<i>FABP1</i>	Pyrosequencing primer	TTGAATTTTAAAGTTTAAGAGATTT
	Forward primer	AATGTTTGGGGTTGTGGGATT
	Reverse primer	Biotin-TTACAAACCCCAACACCCTAA
	Sequence to analyze	TTYGTTTYGGTTTTTTAGGGTGTTGGGGTTGT
miR-411	Pyrosequencing primer	AATATATGATGTAGTAAAATTAGAA
	Forward primer	TAAGGAAGAAATTATGGGTTGTTG
	Reverse primer	Biotin-ATTCCTTCTTTAATACCCAACAAC
	Sequence to analyze	AATAATTATTATATTATGTYGTAATTTTGTGTTGGTATTAAAGAAGGAA

**Table 2.** Primers for pyrosequencing.

## **2.8 RNA extraction from placenta tissues**

Human placenta samples were collected from ERTBB (The Edinburgh Reproductive Tissue Bio Bank) as detailed in Mina et al (2015b). We applied the Qiagen miRNeasy Mini kit (Qiagen, Crawley, West Sussex, UK) protocol, detailed as follows. Approximately 30 mg tissue were cut and mixed with 700 µl QIAzol Lysis Reagent in Magna lyser green beads tubes (Roche, Mannheim, Germany), and homogenized using Qiagen Tissue Lyser for 50 seconds x 25 Hz for 5 minutes and incubated at room temperature for 5 minutes. The lysates were subsequently mixed with 140 µl chloroform and vortexed for 15 seconds. The mixture was incubated at room temperature for 3 minutes, followed by centrifugation at 12,000 xg at 4 °C for 15 minutes. Subsequently the upper aqueous layer (containing RNA) of the samples was transferred to a new tube with 525 µl ethanol added and the mixture was transferred to an RNeasy Mini spin column tube. Three wash steps were followed and the remainder of the extraction steps were as per the manufacturer's protocol.

### **2.8.1 RNA quantification and verification of integrity**

The concentrations of the extracted RNAs were assessed by means of Nanodrop (Thermo-Scientific, Loughborough, UK) and the initial quality control was determined by 260/280 ratio. Samples with total RNA concentration  $\geq 200$  µl were deemed suitable for the subsequent verification stage. The integrity of RNA was then verified using Agilent 6000 Nano kit (Agilent Technologies, Berkshire, UK), which

were processed on the Agilent 2100 Bioanalyzer (Agilent Technologies) as per the manufacturer's protocol. The ribosomal ratio 260/280 results > 2.00 and clear bands in the gel image were used as the cut-off values for excellent RNA integrity.

## **2.9 RT-qPCR**

### **2.9.1 RT-qPCR for FABP1**

DNase treatment was carried out before RT using DNase treatment kit (Promega, Southampton, UK). Briefly, a mixture of RQ1 reaction buffer 10X (1 µl), RQ1 RNase-Free DNase (1 µl) and 7 µl RNA samples was incubated at 37 °C for 30 minutes. RQ1 RNase stop solution (1 µl) was added with another incubation at 65 °C for 10 minutes. Total volume of 20 µl mixture was used for per RT reaction per sample (n=9), including treated RNA mixture (10 µl), 100mM dNTPs with dTTP (0.8 µl), Reverse Transcriptase (50 U/µl, 1 µl), 10 X Reverse Transcription Buffer (2 µl), Nuclease-free water (3.2 µl), primers 10 X (2 µl), according to ABI High Capacity Reverse Transcription Kit (Applied Biosystems, Paisley, UK) protocol. Standard curve was generated with Nuclease-free water for 1:8 dilutions. Mixtures were hold at 30 °C for 16 minutes, followed 30 °C for 42 minutes and 5 °C for 85 minutes, with a final hold at 4 °C by using Prime RT machine.

Total volume of 10 µl mixture was used in qPCR per well, with PerfeCTa<sup>®</sup> QRTPCR Fastmix<sup>®</sup> II (5 µl, Quanta, Bioscience, Leicestershire, UK), three different probes (0.1 µl each, FABP1, TBP, YWHAZ), 0.2 µl forward primer and 0.2 µl reverse

primer (FABP1, TBP, YWHAZ each), Nuclease-free water (2.5 µl) and 2 µl diluted cDNA (1:4). One Nuclease-free water and housekeeping gene were performed in per reaction. All samples were done in triplicates. Plate was hold at 95 °C for 10 minutes, followed 40 cycles at 94 °C for 10 seconds and 68 °C for 60 seconds by using Lighter Cyclor 480 system (Roche, London, UK).

### **2.9.2 RT-qPCR for miR-411**

Total volume of 15 µl mixture was used for per RT reaction per sample (n=46), including 100mM dNTPs with dTTP (0.15 µl), MultiScribe™ Reverse Transcriptase, 50 U/µl (1 µl), 10 X Reverse Transcription Buffer (1.5 µl), RN-ase Inhibitor, 20 U/µl (0.19 µl), Nuclease-free water (4.16 µl), U6 primers (3 µl), miR-411 primers (3 µl) and diluted RNA samples (1 ng, 5 µl), by following the protocol of Taqman miRNA reverse transcription kit (Applied Biosystems, UK). Standard curve was made by Nuclease-free water for 1:8 dilutions. Mixtures were hold at 16 °C for 30 minutes, followed 42 °C for 30 minutes and 85 °C for 5 minutes, with a final hold at 4 °C by using Prime RT machine.

Total volume of 10 µl mixture was used in qPCR per well, with PerfeCTa® QRTPCR Fastmix® II (5 µl, Quanta, Bioscience, Leicestershire, UK), 20X miR-411 assay (ThermoFisher scientific, UK), 20X U6 assay (ThermoFisher scientific, UK, ID: 001973), Nuclease-free water (2.5 µl) and 2 µl diluted cDNA (1:5). One Nuclease-free water and one mixture without MultiScribe™ Reverse Transcriptase



were used as negative control. All samples were done in triplicates. Plate was hold at 95 °C for 10 minutes, followed 40 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds by using Lighter Cyclor 480 (Roche, London, UK). Two miR-411 assays: miR-411-5p (ID: 001610) and miR-411-3p (ID: 00002238) were tested in this study, details see Table 3. MiR-411-5p and miR-411-3p arise from 5' arm and 3' arm of the miR-411 hairpin respectively, and it has been demonstrated in a previous study that miRNAs derived from different arms show different mRNA levels and functions (Guo *et al.*, 2015; Kuo *et al.*, 2015). Thus we chose to test both of these two miRNAs to investigate the gene expression of miR-411 in placenta.

Assay name	Sequence
miR-411-5p	(5'-3') UAGUAGACCGUAUAGCGUACG
miR-411-3p	(3'-5') UAUGUAAACACGGUCCACUAACC
U6	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGA ACGATACAGAGAAGATTAGCATGGCCCCTGCGCA AGGATGACACGCAAATTCGTGAAGCGTTCCATATT TT

**Table 3.** Assays used in qPCR reaction in this study.

## **2.10 Data analysis**

Data generated from qPCR were analyzed by SPSS version 22 (IBM, New York, USA). Data normal distribution was tested by Shapiro-Wilk's test ( $P > 0.05$  as significant), Q-Q Plot and visual histograms. Outliers ( $>3SD$  from mean) were excluded. We used  $\ln$ -transformation to normalize data that were not normally distributed but this did not improve the distribution. Therefore parametric statistics were used for normally distributed data and non-parametric statistics for non-normally distributed data. T-test (unpaired) was used to compare the differences in DNA methylation data between obese and lean groups. Mann-Whitney U test was used to compare gene expression data between groups. Pearson's correlation was used to test correlations of DNA methylation data with maternal BMI, smoking status, fetal birth weight and infant BMI etc. and Spearman correlation was used to test correlations of gene expression data with maternal BMI, smoking status, fetal birth weight and infant BMI etc. Regression analyses were performed with mRNA levels as independent variables and different maternal and fetal status (such as maternal smoking status and fetal sex) as dependent variables. Histograms of residuals were tested as normal distribution before performing regression analysis.  $P < 0.05$  was defined as significant. Figures were drawn using Graphpad 6 (USA).

## **3 Results**

### **3.1 The demographics of participants**

Characteristics of the participants from which the placental samples were used in this study are shown in Table 4. There were significant differences in maternal BMI, age and Standard Deviation Score of birth weight (sds\_bw) between lean and obese group. Maternal BMI was significantly higher in obese group than lean group, while maternal age was significantly higher in lean group compared with obese group. Sds\_bw in obese group was significantly higher than in the lean group.

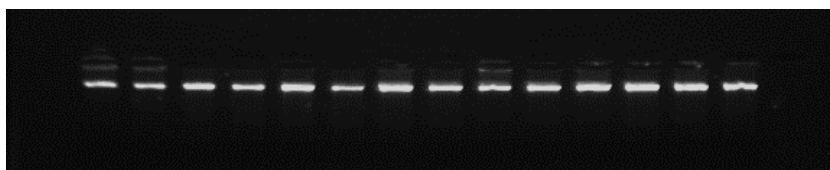
Demographics	Lean (n=29)	SO (n=31)	P value
Maternal BMI, kg/m <sup>2</sup> , mean (S.D.)	22.9 (1.6)	44.4 (3.8)	<b>P&lt;0.001<sup>a</sup></b>
Age, years, mean (S.D.)	34.1 (4.6)	31.4 (4.8)	<b>P&lt;0.05<sup>a</sup></b>
Smoking, n (%)	2 (6.9)	5 (16.1)	
Ex-smoker, n (%)	16 (55.2)	7 (22.6)	<b>P&lt;0.05<sup>b</sup></b>
Non-smoking, n (%)	11 (37.9)	19 (61.3)	
Gestational age, days, mean (S.D.)	281.6 (10.3)	283.2 (9.3)	P>0.05 <sup>a</sup>
Infant BMI, kg/m <sup>2</sup> , mean (S.D.)	12.3 (1.2)	11.7 (2.1)	P>0.05 <sup>a</sup>
Birth weight, g, mean (S.D.)	3454 (429.9)	3533 (711.7)	P>0.05 <sup>a</sup>
Male baby, n (%)	14 (48.3)	14 (45.2)	
Female baby, n (%)	15 (51.7)	17 (54.8)	P>0.05 <sup>b</sup>
SDS_BMI, score, mean (S.D.)	-1.0 (1.0)	-1.3 (1.5)	P>0.05 <sup>a</sup>
SDS_bw, score, mean (S.D.)	0.03 (0.9)	0.5 (1.3)	<b>P=0.05<sup>a</sup></b>
SDS_PI, kg/m <sup>3</sup> , mean (S.D.)	2.3 (0.3)	2.1 (0.4)	P>0.05 <sup>a</sup>

**Table 4.** Maternal and fetal demographics. SO, severely obese; S.D., standard deviation; BMI, body mass index; SDS\_bw, Standard Deviation Score of birth weight (British standard), this is birth weight index adjusted for their gestational age and sex; PI, Ponderal index in  $\text{kg/m}^3$ , this is BMI version for infants as infants tend to have similar length of leg and torso, unlike adults; SDS\_BMI, Standard Deviation Score of birth BMI (British standard), there is no national standard for PI. “a” represents P value generated using t-test (significant  $<0.05$ ). “b” represents P value generated using Fisher’s exact test (significant  $<0.05$ ).

### **3.2 DNA quality and quantity**

The quality of DNA samples was measured by Nanodrop, with concentration at 400 ~ 500 µl, 260/280 at 2.0, 260/230 at 1.8. The integrity of DNA was measured by 1% agarose gel with images show one clean band and no smear. An example is given in Figure 4.

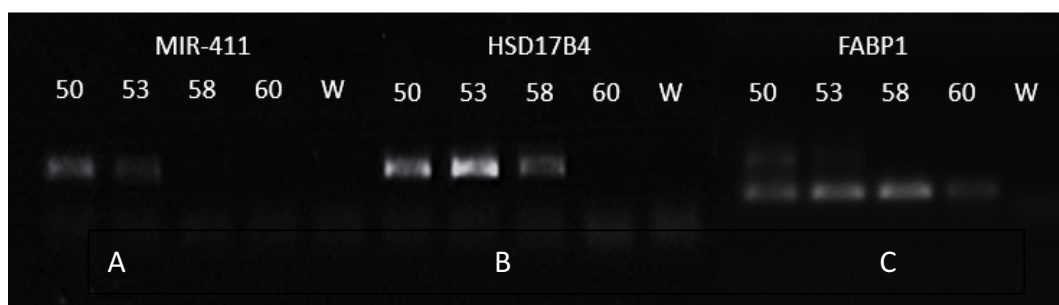




**Figure 4.** An example of gel image of DNA samples. From left to right sample (no.): 645, 572, 563, 628, 575, 625, 634, 631, 411, 403, 447, 442, 626, 646.

### **3.3 Primer test**

Different primers for each gene were tested before pyrosequencing. Primers were adjusted at different temperature while running PCR, gel image was captured. In Figure 5. Examples were given for each gene of primer test. From the image we can see, miR-411 assay showed a brightest band at 50 °C (Fig.5 A), while a brightest band was shown at 58 °C for FABP1 assay (Fig.5 B), and HSD17B4 assay presented best results at 53 °C (Fig.5 C).

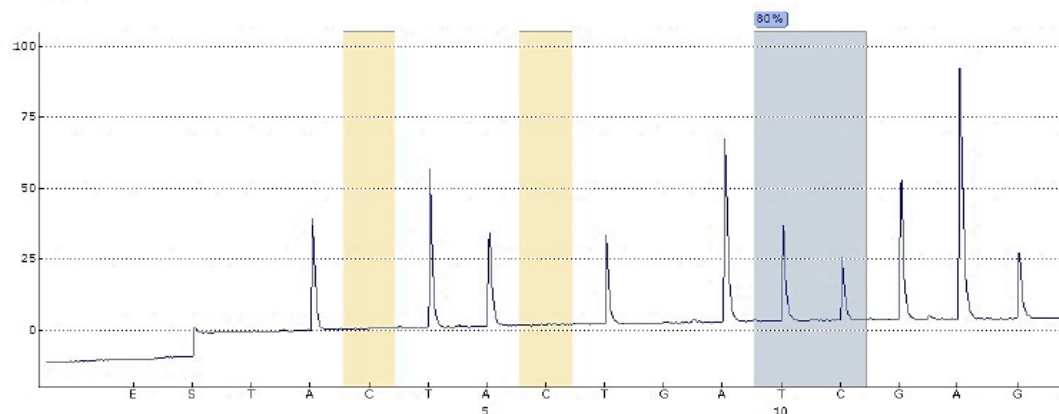


**Figure 5.** PCR gel images of test primers in different temperature. A. shows a clearer band at 50 °C; B. shows a brighter band at 53 °C, and in C. a clean band can be observed at 58 °C. W means water control.

### **3.4 Pyrosequencing for DNA methylation and data analysis**

There are three different results from pyrosequencing, 'pass' (blue), 'check' (yellow) and 'fail' (red), both 'check' and 'fail' results were repeated in this experiment by improving the quality of DNA samples. Water controls showed as 'fail' status means no contamination. Examples of pyrosequencing results were shown in Figure 6.

Assay: Hsd17b4-as1  
Sample ID: dna  
Note: 154  
Analysis version: 1.0.10



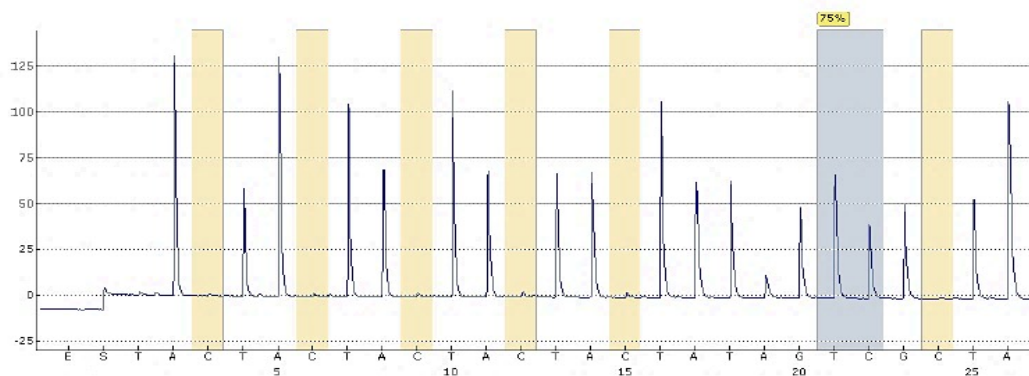
Sequence to analyze:  
ATTATAATYGGAAAGTAGAAATTATTTATTGGA

Position	1
Quality	Passed
Meth (%)	80

No warnings.

(A)

Assay: mir-411 assay4  
Sample ID: 926  
Note: dna  
Analysis version: 1.0.10



Sequence to analyze:  
AATAATTATTATATTATGTGTGTAAATTTGTGTGGGTATTAAAGAAGGAA

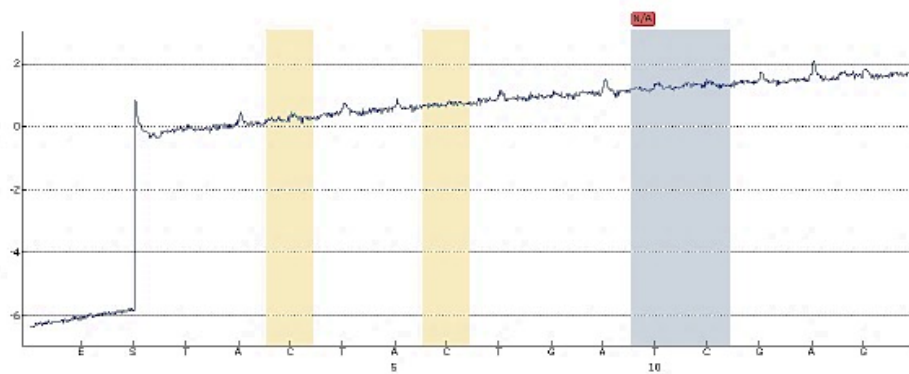
Position	1
Quality	Check
Meth (%)	75

General warnings:  
Uncertain bisulfite conversion at dispensation: 15.

Warnings:  
No additional

(B)

Assay: Hsd17b4-as1  
Sample ID: water  
Note:  
Analysis version: 1.0.10



Sequence to analyze:  
ATTATAATYGGAAAGTAGAAATTATTTATTGGA

Position	1
Quality	N/A
Meth (%)	-

General warnings:  
Not analyzable due to lack of data (overall).

Warnings:  
No additional

(C)

**Figure 6.** Examples of pyrosequencing results. (A) A passed (blue) result with 80% DNA methylation and no additional warning. (B) A need to be checked (yellow) result with 75% DNA methylation with uncertain bisulfite conversion at position 15. (C) A failed (red) result with no data can be analyzed.

Differences in DNA methylation between the three genes: miR-411, *FABP1* and *HSD17B4* are shown in Table 5. DNA methylation of both *FABP1* and miR-411 was significantly higher in obese compared with lean but there were no differences in DNA methylation of *HSD17B4* between groups.

Gene	Lean/Obese	N	Mean(%)	Std. Deviation	P-value
<i>HSD17B4</i>	lean	28	73.17	6.60	0.62 <sup>a</sup>
	obese	30	74.13	7.85	
<i>miR-411</i>	lean	29	58.96	15.84	<b>0.01<sup>a</sup></b>
	obese	31	68.96	13.44	
<i>FABP1</i>	lean	27	85.88	7.16	<b>0.01<sup>a</sup></b>
	obese	27	89.74	2.76	

**Table 5.** Differences between obese and lean in placental DNA methylation in three different genes: HSD17B4, miR-411 and FABP1.  
“a” represents P value generated using t-test (significant <0.05).



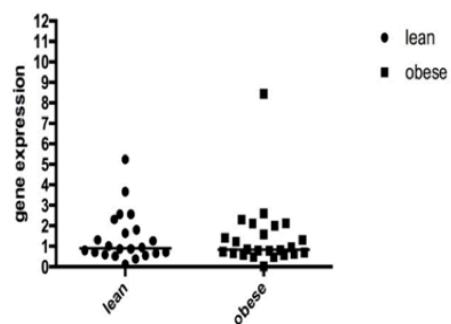
### **3.5 RT-qPCR data analysis with DNA methylation results**

#### **3.5.1 miR-411-5p and miR-411-3p gene expression in lean and obese placenta overview**

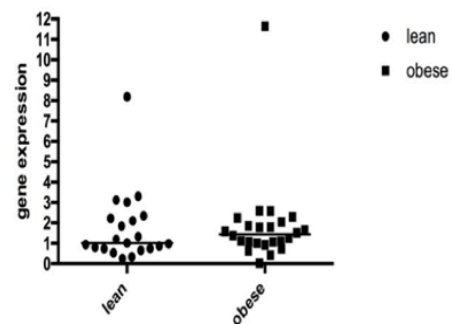
The miR-411-5p gene expression in lean and obese placentas is shown in Fig.7 (a), and miR-411-3p is shown in Fig.7 (b). No differences were observed in either miR-411-5p or miR-411-3p mRNA levels between lean and obese groups.

miR-411-5p gene expression in lean and obese placenta

miR-411-3p-gene expression in lean and obese placenta



(a)



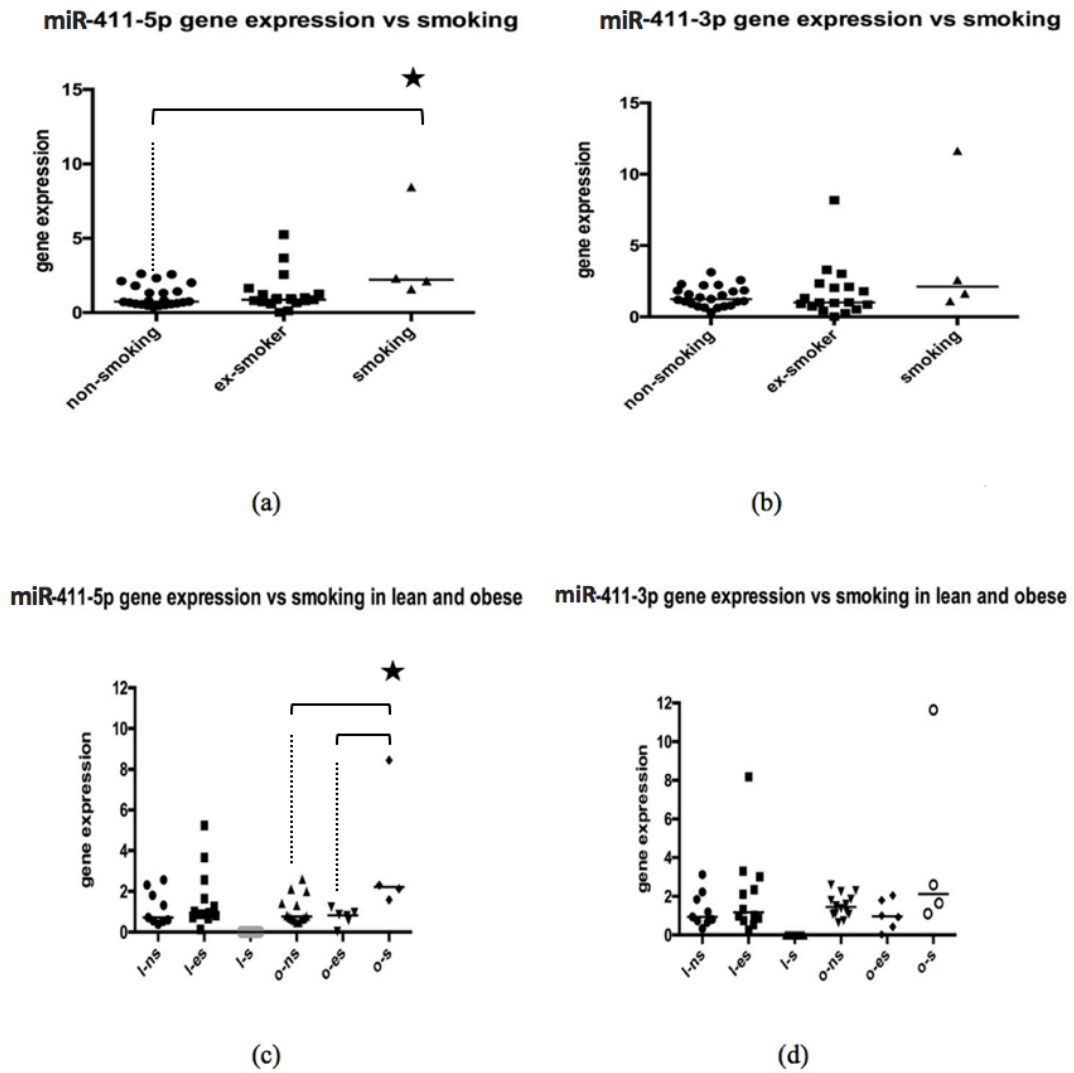
(b)

**Figure 7.** Scatter plot shows miR-411-5p/3p gene expression in lean/obese placenta. (a) miR-411-5p gene expression in lean (n=22) and obese (n=24) placentas and (b) miR-411-3p gene expression in lean (n=21) and obese (n=24) placentas. The horizontal line is presented as median.

#### **3.5.1.1 miR-411-5p and miR-411-3p gene expression vs maternal smoking status**

The mRNA levels of miR-411-5p/3p were compared with maternal smoking status (Figure 8). Gene expression of miR-411-5p in placentas from smoking group was higher in samples collected from ex-smokers and significantly higher than samples from non-smoking women ( $P=0.03$ , see Fig.8 (a)). A similar pattern was observed in miR-411-3p, though there were no statistical differences (Fig.8 (b)).

Further investigation of gene expression differences between lean and obese placentas vs maternal smoking status are shown in Fig.8 (c) & (d). We can see that obese placentas in smoking group showed the highest mRNA levels of miR-411-5p (Fig.8 (c)), which was significantly higher than non-smoking obese placentas ( $P=0.04$ ) and ex-smoker obese placentas ( $P=0.03$ ), but there were no differences comparing lean placentas ( $P>0.05$ ). The analysis of miR-411-3p also showed a similar pattern but there were no significant differences (Fig.8 (d)). The mRNA levels in obese ex-smoker were higher than obese non-smoking in miR-411-5p group, whereas the mRNA levels in obese ex-smoker was lower than obese non-smoking in miR-411-3p group. No lean placenta samples were collected from smoking group in this study.



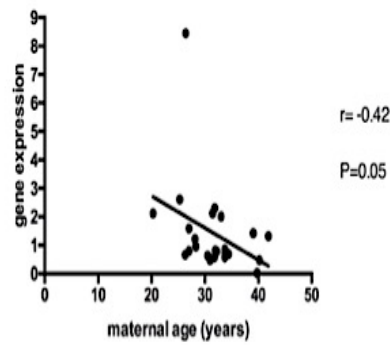
**Figure 8.** Scatter plot shows miR-411-5p/3p gene expression vs smoking in lean and obese placenta. (a) Overview of miR-411-5p gene expression in placentas vs maternal smoking status (non-smoking=23, ex-smoker=19, smoking=4) and (b) Overview of miR-411-3p gene expression in placentas vs maternal smoking status (non-smoking=23, ex-smoker=18, smoking=4). (c) miR-411-5p gene expression in lean and obese placentas vs maternal smoking status respectively. (d) miR-411-3p gene expression in lean and obese placentas vs maternal smoking status respectively. The horizontal line is presented as median.

**Note:** ★P<0.05. l-ns=lean non-smoking, l-es=lean ex-smoker, l-s=lean smoking, o-ns=obese non-smoking, o-es=obese ex-smoker, o-s=obese smoking. L-s group (n=0).

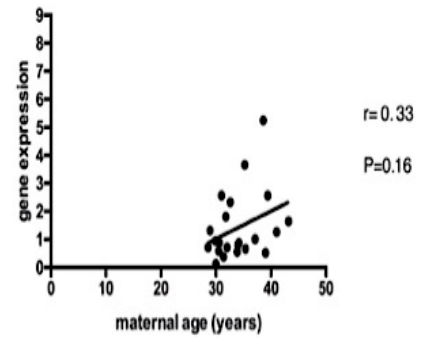
### **3.5.1.2 miR-411-5p and miR-411-3p gene expression vs maternal age and BMI**

The correlation of miR-411-5p/3p gene expression with maternal age and BMI was tested. There was a negative correlation between miR-411-5p mRNA levels and maternal age in obese placentas ( $r=-0.42$ ,  $P=0.05$ ), but no correlation was seen in lean placentas between obese placentas (Fig.9 (a,b)). There was no correlation of miR-411-3p mRNA levels with maternal age in either lean or obese groups (Fig.9 (c,d)). There was no correlation of miR-411-5p gene expression with maternal BMI, or with miR-411-3p gene expression (Fig.9 (e,f)).

miR-411-5p gene expression vs maternal age in obese placenta    miR-411-5p gene expression vs maternal age in lean placenta

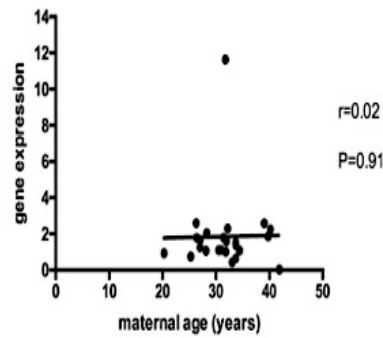


(a)

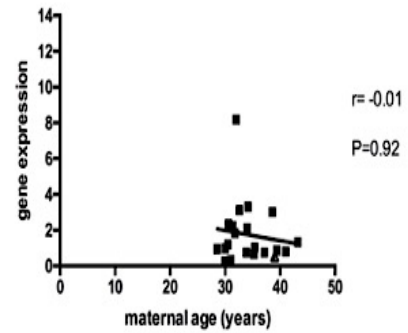


(b)

miR-411-3p gene expression vs maternal age in obese placenta    miR-411-3p gene expression vs maternal age in lean placenta

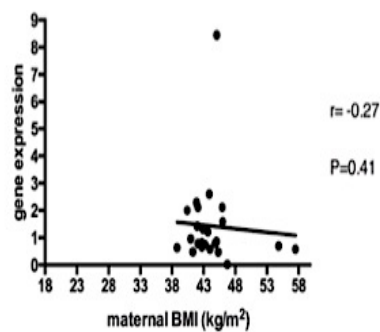


(c)

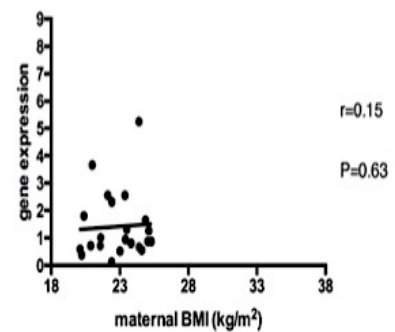


(d)

miR-411-5p gene expression vs maternal BMI in obese placenta    miR-411-5p gene expression vs maternal BMI in lean placenta



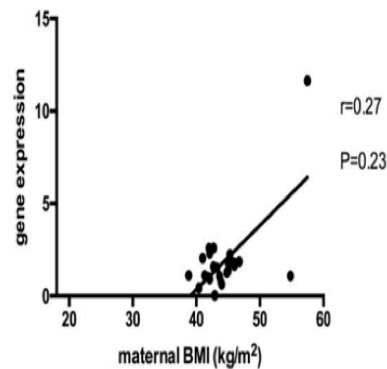
(e)



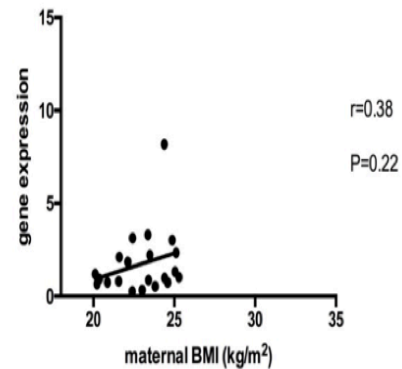
(f)

miR-411-3p gene expression vs maternal BMI in obese placenta

miR-411-3p gene expression vs maternal BMI in lean placenta



(g)



(h)

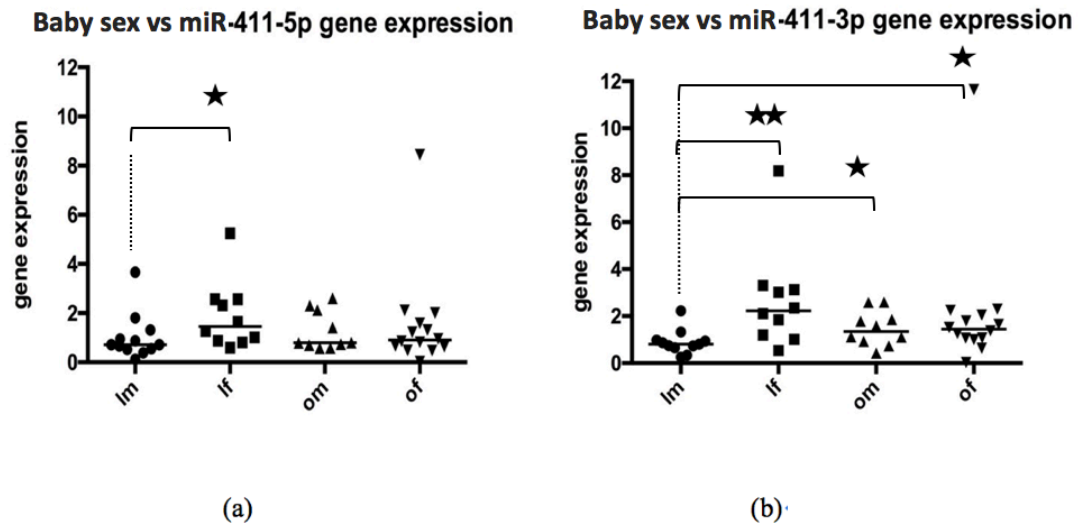
**Figure 9.** Correlation between miR-411-5p/3p gene expression and maternal age/BMI. (a) miR-411-5p gene expression in obese placentas vs maternal age. (b) miR-411-5p gene expression in lean placentas vs maternal age. (c) miR-411-3p gene expression in obese placentas vs maternal age. (d) miR-411-3p gene expression in lean placentas vs maternal age. (e) miR-411-5p gene expression in obese placentas vs maternal BMI. (f) miR-411-5p gene expression in lean placentas vs maternal BMI. (g) miR-411-3p gene expression in obese placentas vs maternal BMI. (h) miR-411-3p gene expression in lean placentas vs maternal BMI.

**Note:** Spearman correlation indexes “r” and “P” (significant <0.05) are shown in the figure. The straight line represents the best-fit line generated by liner regression analysis.

### **3.5.1.3 miR-411-5p and miR-411-3p gene expression vs baby sex**

Female lean placenta showed the highest mRNA levels in both miR-411-5p and miR-411-3p. The gene expression of miR-411-5p in female lean placenta was significantly higher than male lean placenta group ( $P=0.04$ ). MiR-411-5p gene expression was also higher in male obese placenta than male lean placenta but this difference was not statistically significant (Fig.10 (a)). Similar results also can be seen in miR-411-3p, the mRNA level was noticeably higher in female lean placenta than male lean placenta group ( $P=0.004$ ). We also found miR-411-3p gene expression in male obese group was significantly higher than male lean group ( $P=0.05$ ). Moreover, miR-411-3p gene expression in female obese group was also significantly higher than male lean group ( $P=0.01$ , Fig.10 (b)).





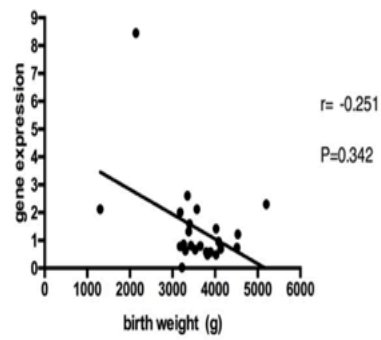
**Figure 10.** Scatter plot shows miR-411-5p/3p gene expression vs baby sex in lean and obese placenta. (a) miR-411-5p gene expression in lean and obese placentas vs baby sex. (b) miR-411-3p gene expression in lean and obese placentas vs baby sex.

**Note:** ★ $P < 0.05$ ; ★★  $P < 0.005$ . lm=lean male, lf=lean female, om=obese male, of=obese female.

#### **3.5.1.4 miR-411-5p and miR-411-3p gene expression vs birth weight and infant BMI.**

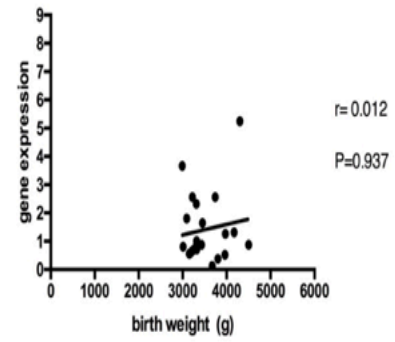
We found there were no correlations of infant birth weight with miR-411-5p and miR-411-3p gene expression in lean and obese placentas (Fig.11 (a,b,c,d)). We found a strong positive correlation between infant BMI and the mRNA levels of miR-411-5p in lean group ( $r=0.636$ ,  $P<0.005$ , Fig.11 (f)), and similar results were also seen in miR-411-3p ( $r=0.661$ ,  $P<0.005$ , Fig.11 (h)). However, no correlation of infant BMI and gene expression was observed in miR-411-5p/3p in obese placentas (Fig.11 (e,g)).

miR-411-5p gene expression vs birth weight in obese placenta



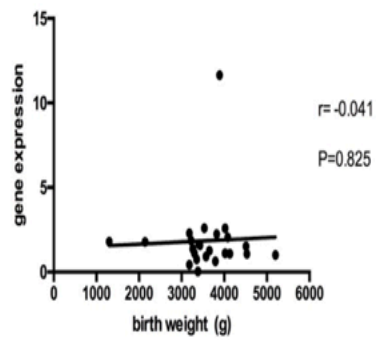
(a)

miR-411-5p gene expression vs birth weight in lean placenta



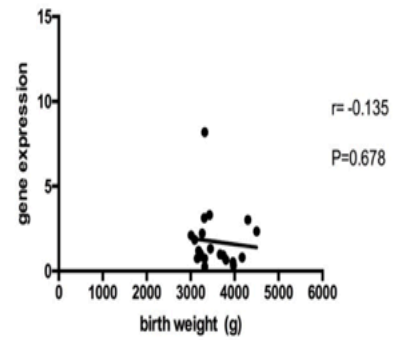
(b)

miR-411-3p gene expression vs birth weight in obese placenta



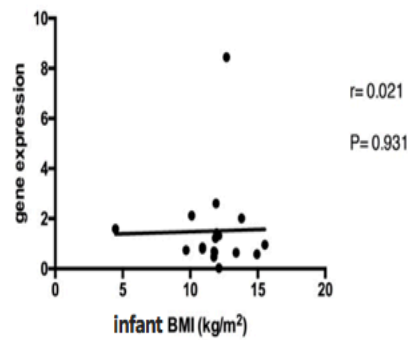
(c)

miR-411-3p gene expression vs birth weight in lean placenta



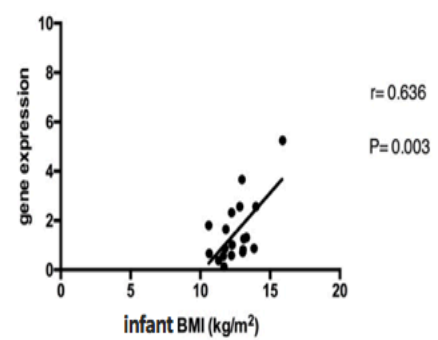
(d)

miR-411-5p gene expression vs infant BMI in obese placenta



(e)

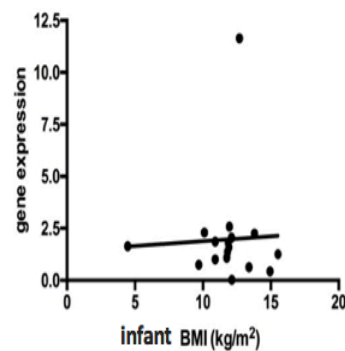
miR-411-5p gene expression vs infant BMI in lean placenta



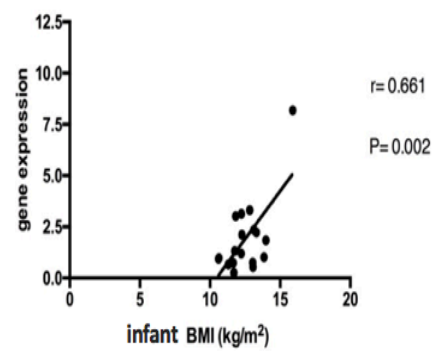
(f)

Infant BMI vs miR-411-3p gene expression in obese placenta

Infant BMI vs miR-411-3p gene expression in lean placenta



(g)



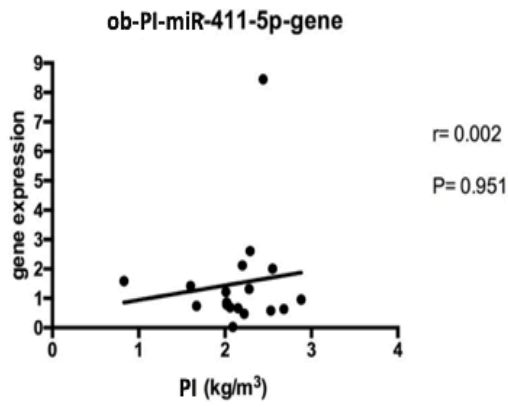
(h)

**Figure 11.** Correlation between miR-411-5p/3p gene expression and infant birth weight/BMI. (a) miR-411-5p gene expression in obese placentas vs birth weight. (b) miR-411-5p gene expression in lean placentas vs birth weight. (c) miR-411-3p gene expression in obese placentas vs birth weight. (d) miR-411-3p gene expression in lean placentas vs birth weight. (e) miR-411-5p gene expression in obese placentas vs infant BMI. (f) miR-411-5p gene expression in lean placentas vs infant BMI. (g) miR-411-3p gene expression in obese placentas vs infant BMI. (h) miR-411-3p gene expression in lean placentas vs infant BMI.

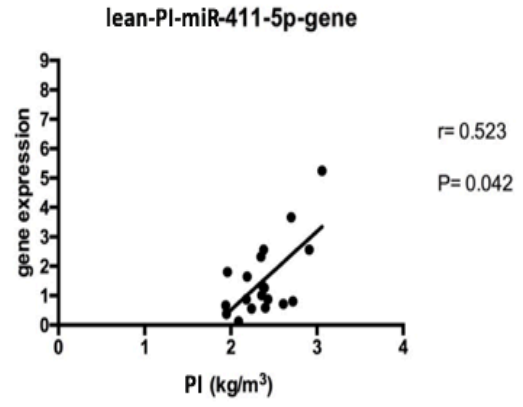
**Note:** Spearman correlation indexes “r” and “P” (significant <0.05) are shown in the figure. The straight line represents the best-fit line generated by liner regression analysis.

### **3.5.1.5 miR-411-5p and miR-411-3p gene expression vs PI and sds\_BMI/bw**

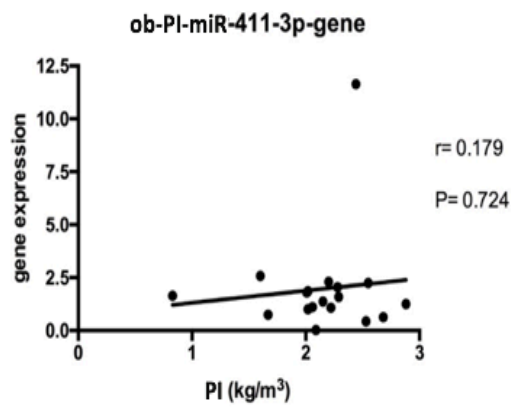
To further investigate the correlation of miR-411-5p/3p gene expression with infant birth weight and BMI, we also analyzed PI (ponderal index in  $\text{kg/m}^3$ , a BMI version for infants as infants tend to have similar length of leg and torso, unlike adults), sds-bw (Standard Deviation Score of birth weight (British standard), birth weight index adjusted for their gestational age and sex) and sds\_BMI (Standard Deviation Score of birth BMI (British standard)). Although there was no correlation of PI with miR-411-5p/3p gene expression in obese placentas (Fig.12 (a,c)), there was a significant positive correlation of PI with miR-411-5p/3p gene expression in lean placentas (5p:  $r=0.523$ , 3p:  $r=0.541$ ;  $P<0.05$ , Fig.12 (b,d)). We also found a significantly positive correlation of sds\_BMI with mRNA levels of miR-411-5p/3p in lean group (Fig.12 (f,h)), whereas no correlation was seen in obese group (Fig.12 (e,g)). No correlation was shown between sds\_bw and miR-411-5p/3p gene expression in both groups (Fig.12 (i,j,k,l)). Sds\_bw in lean placentas tended to be negatively correlated with miR-411-3p mRNA levels ( $r=-0.431$ ,  $P=0.062$ , (Fig.12 (l))).



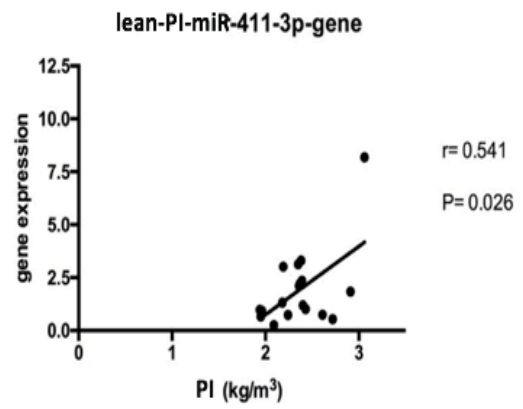
(a)



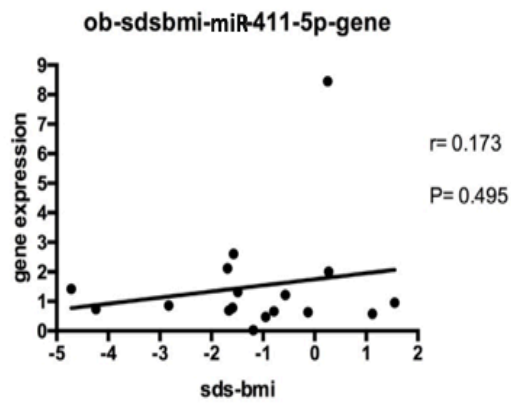
(b)



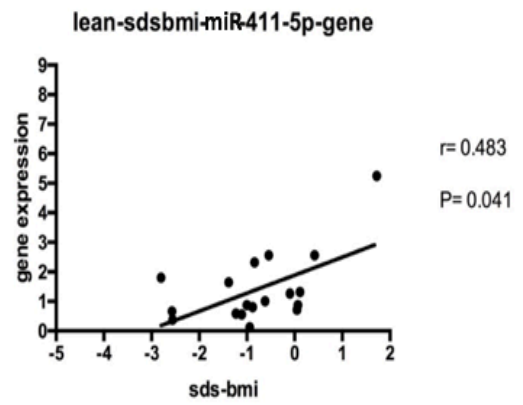
(c)



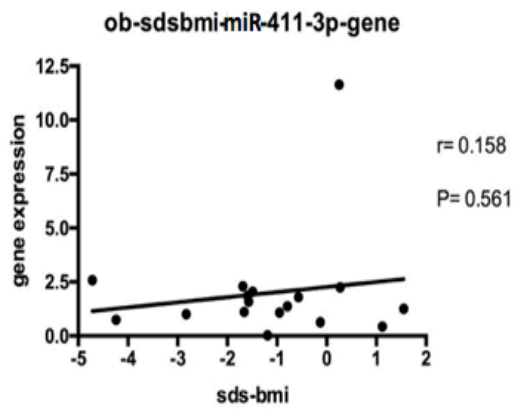
(d)



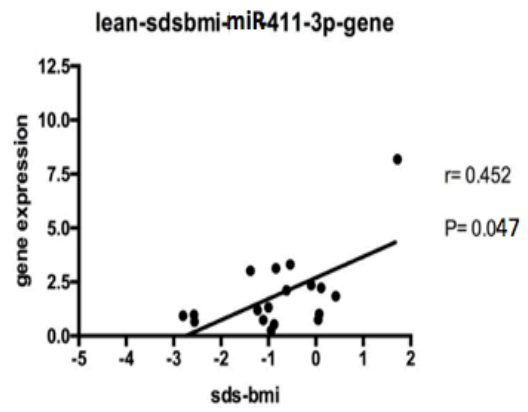
(e)



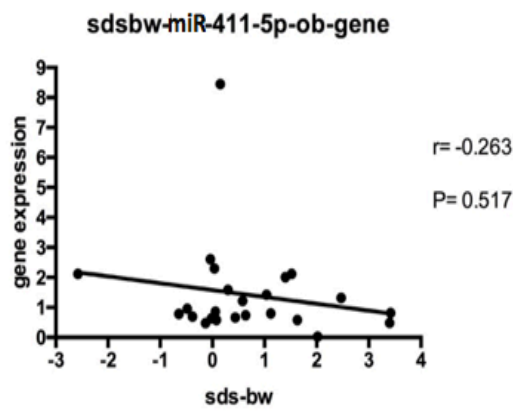
(f)



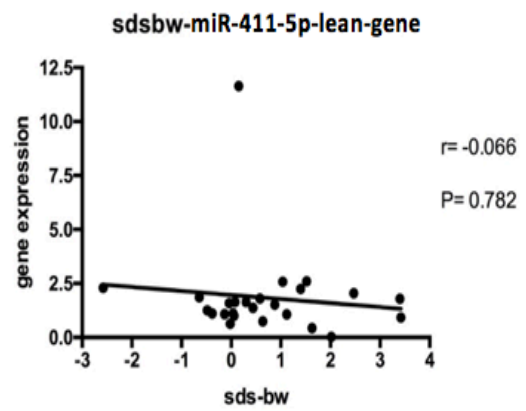
(g)



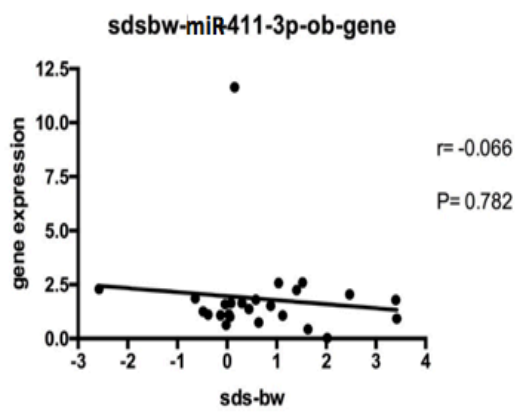
(h)



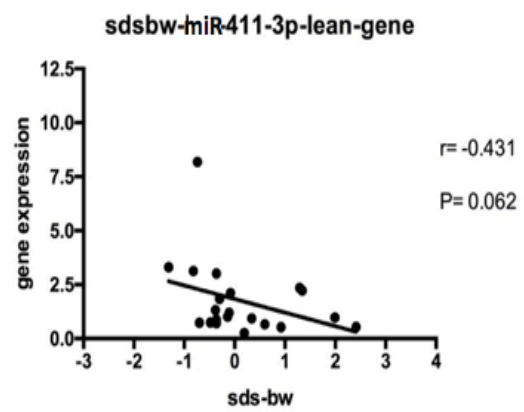
(i)



(j)



(k)



(l)

**Figure 12.** Correlation between miR-411-5p/3p gene expression and PI/sds\_bw/sds\_BMI. (a) miR-411-5p gene expression in obese placentas vs PI. (b) miR-411-5p gene expression in lean placentas vs PI. (c) miR-411-3p gene expression in obese placentas vs PI. (d) miR-411-3p gene expression in lean placentas vs PI. (e) miR-411-5p gene expression in obese placentas vs sds\_BMI. (f) miR-411-5p gene expression in lean placentas vs sds\_BMI. (g) miR-411-3p gene expression in obese placentas vs sds\_BMI. (h) miR-411-3p gene expression in lean placentas vs sds\_BMI. (i) miR-411-5p gene expression in obese placentas vs sds\_bw. (j) miR-411-5p gene expression in lean placentas vs sds\_bw. (k) miR-411-3p gene expression in obese placentas vs sds\_bw. (l) miR-411-3p gene expression in lean placentas vs sds\_bw.

**Note:** Spearman correlation indexes “r” and “P” (significant <0.05) are shown in the figure. The straight line represents the best-fit line generated by liner regression analysis.



### **3.5.1.6 Regression of miR-411-5p and miR-411-3p gene expression vs maternal smoking status and baby sex**

Multiple linear regression analyses were performed to examine whether the relationship between miR-411-5p/3p gene expressions and baby sex was independent of maternal smoking, see Table 6. Analyses were conducted separately for the lean and obese groups. In the lean group, miR-411-5p gene expressions was higher in female placentas than in male placentas, independently of maternal smoking (Table 6a). In contrast, in obese (Table 6b), there was no association of miR-411-5p gene expression with fetal sex, but smoking was associated with this gene. MiR-411-5p gene expressions was higher in smokers than ex-smokers and non-smoking in obese placentas. There were similar findings for miR-411-3p in lean (Table 6c) and a similar pattern in obese (Table 6d), though the latter failed to reach statistical significance.

**The association of miR-411-5p gene expression(lean) vs independent variable<sup>a</sup>**

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
Constant	-1.256	1.369		-.917	.371	-4.132	1.621
Maternal smoking <sup>a</sup>	.315	.712	.091	.442	.664	-1.181	1.810
Fetal_sex <sup>a</sup>	1.694	.705	.497	2.402	<b>.027</b>	.212	3.176

(a). Dependent Variable: miR-411-5p\_lean\_gene expression

**The association of miR-411-5p gene expression(obese) vs independent variable<sup>a</sup>**

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
Constant	-.642	1.228		-.523	.607	-3.197	1.912
Maternal smoking <sup>a</sup>	.997	.406	.471	2.458	<b>.023</b>	.153	1.841
Fetal_sex <sup>a</sup>	.309	.625	.095	.495	.626	-.990	1.608

(b). Dependent Variable: miR-411-5p\_obese\_gene expression

**The association of miR-411-3p gene expression(lean) vs independent variable<sup>a</sup>**

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
Constant	-1.256	1.369		-.917	.371	-4.132	1.621
Maternal smoking <sup>a</sup>	.315	.712	.091	.442	.664	-1.181	1.810
Fetal_sex <sup>a</sup>	1.694	.705	.497	2.402	<b>.027</b>	.212	3.176

(c). Dependent Variable: miR-411-3p\_lean\_gene expression

**The association of miR-411-3p gene expression(obese) vs independent variable<sup>a</sup>**

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
Constant	-.874	1.710		-.511	.615	-4.430	2.682
Maternal smoking <sup>a</sup>	1.045	.565	.370	1.851	.078	-.129	2.219
Fetal_sex <sup>a</sup>	.674	.870	.155	.775	.447	-1.135	2.482

(d). Dependent Variable: miR-411-3p\_obese\_gene expression

**Table 6.** Multiple linear regression analysis. (a) regression between miR-411-5p\_lean gene expression and maternal smoking status/fetal sex. (b) regression between miR-411-5p\_obese gene expression and maternal smoking status/fetal sex. (c) regression between miR-411-3p\_lean gene expression and maternal smoking status/fetal sex. (d) regression between miR-411-3p\_obese gene expression and maternal smoking status/fetal sex.

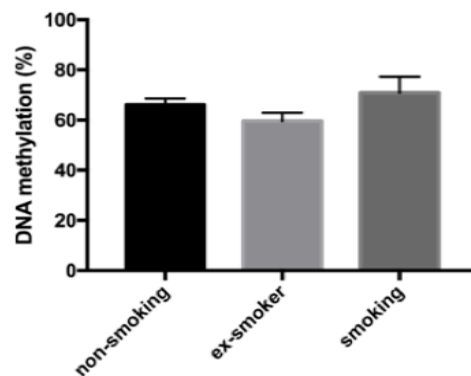
**Note:**  $P < 0.05$  as significant. a: independent variable (maternal smoking status and fetal sex).

### **3.5.2 miR-411 DNA methylation in lean and obese placenta vs maternal smoking status**

There were no substantial differences in miR-411 DNA methylation between smoking, ex-smoker and non-smoking groups (Fig.13 (a)).

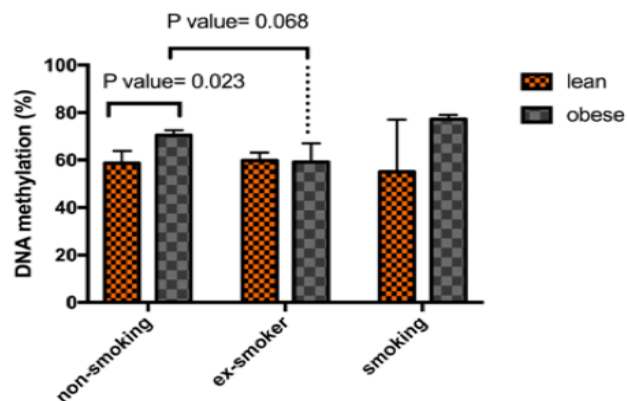
In detailed analysis between lean and obese groups with different smoking status is shown in Fig.13 (b). The mean DNA methylation percentage of miR-411 in obese non-smoking group was higher than the ex-smoker in the same group but not significant different ( $P>0.05$ ), whereas the mean DNA methylation percentage of miR-411 in obese non-smoking group was significantly higher compared with lean non-smoking group ( $P<0.05$ ).

### miR-411 DNA methylation vs smoking in lean and obese placenta



(a)

### Smoking vs miR-411 DNA methylation in lean and obese placenta



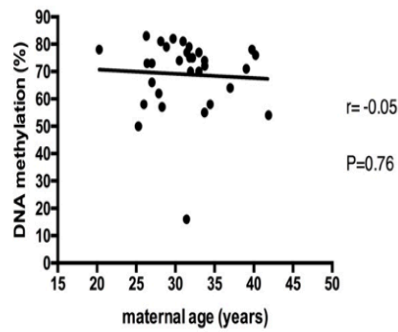
(b)

**Figure 13.** Bar chart shows miR-411 DNA methylation vs smoking in lean and obese placenta. (a) Overview of miR-411 DNA methylation in all placenta samples vs maternal smoking status (non-smoking=30, ex-smoker=23, smoking=7) and (b) Overview of miR-411 DNA methylation in lean and obese placentas respectively vs maternal smoking status. Data are presented as mean value of percentage.

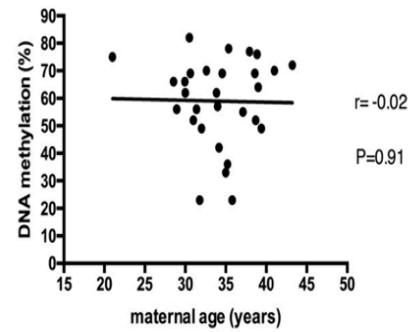
### **3.5.2.1 miR-411 DNA methylation in lean and obese placenta vs maternal age**

We found there was no correlation of miR-411 DNA methylation with maternal age in either lean or obese group (Figure 14).

miR-411 DNA methylation vs maternal age in obese placenta      miR-411 DNA methylation vs maternal age in lean placenta



(a)



(b)

**Figure 14.** Correlation between miR-411 DNA methylation and maternal age. (a) miR-411 DNA methylation in obese placentas(n=31) vs maternal age. (b) miR-411 DNA methylation in lean placentas(n=29) vs maternal age.

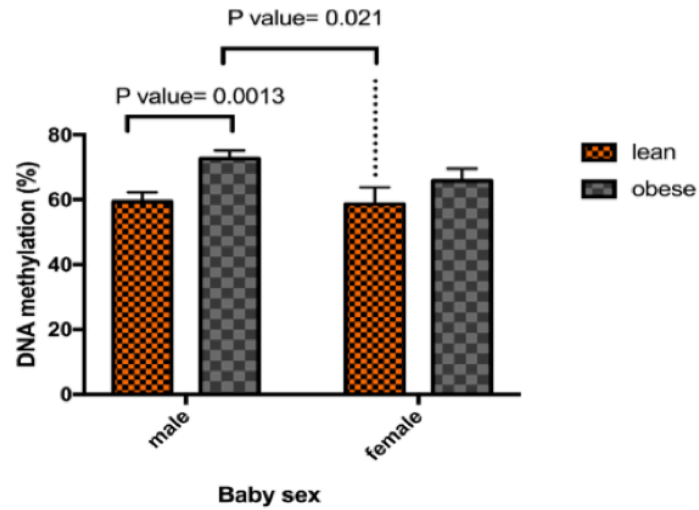
**Note:** Pearson correlation indexes “r” and “P” (significant <0.05) are shown in the figure. The straight line represents the best-fit line generated by liner regression analysis.



### **3.5.2.2 miR-411 DNA methylation in lean and obese placentas vs baby sex**

The percentage of miR-411 DNA methylation in male baby obese group showed the highest level among all samples. In detail, miR-411 DNA methylation was significantly higher in male obese group than male lean group ( $P<0.05$ ). In female group, female baby in obese group also showed higher DNA methylation than female lean group, but no different. The DNA methylation in male infant obese group was also significantly higher by comparing with female baby in lean group ( $P<0.05$ ). See Figure 15.

# **Baby sex vs miR-411 DNA methylation in lean and obese placenta**



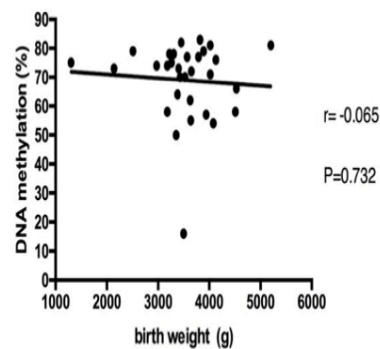
**Figure 15.** Bar chat showing overview of miR-411 DNA methylation in lean and obese placentas respectively vs baby sex. (male obese=14, male lean=14, female obese=17, female lean=15). Data are presented as mean value of percentage.

### **3.5.2.3 miR-411 DNA methylation in lean and obese placenta vs birthweight, infant BMI and PI/sds\_bw/sds\_BMI**

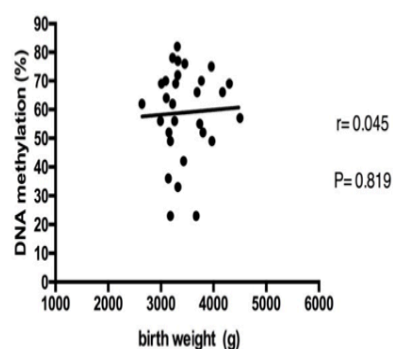
No correlation was detected between miR-411 DNA methylation with infant birth weight, BMI, sds\_bw and sds\_BMI in both lean and obese placentas. There was a significant negative correlation between miR-411 DNA methylation with PI in obese group ( $r=-0.491$ ,  $P<0.05$ ). See Fig. 16 (i).

miR-411 DNA methylation vs fetal birth weight in obese placenta

miR-411 DNA methylation vs fetal birth weight in lean placenta

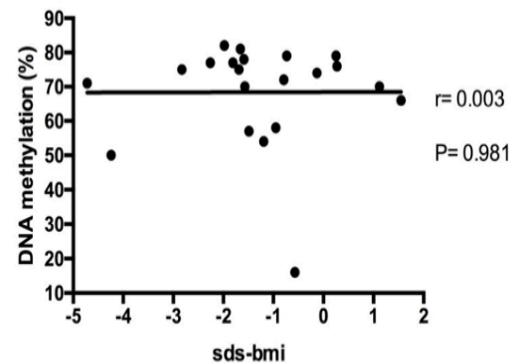


(a)



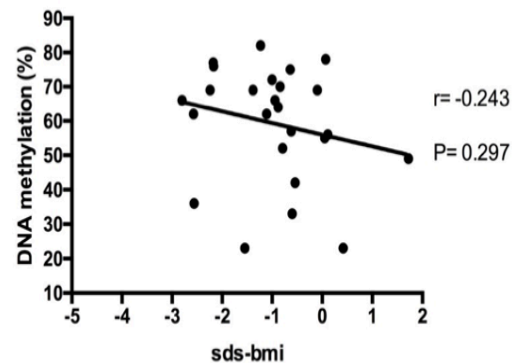
(b)

ob-sdsbmi-DNA methylation

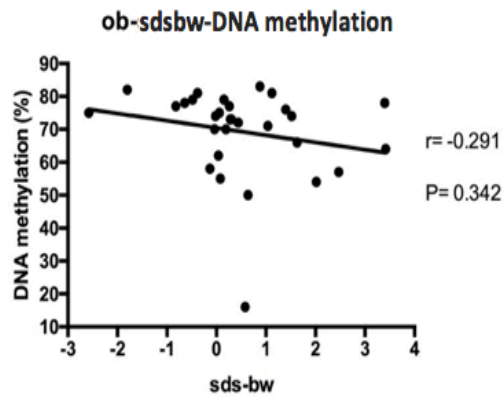


(c)

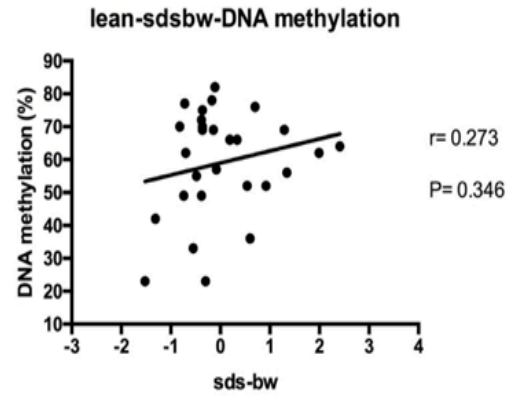
lean-sdsbmi-DNA methylation



(d)

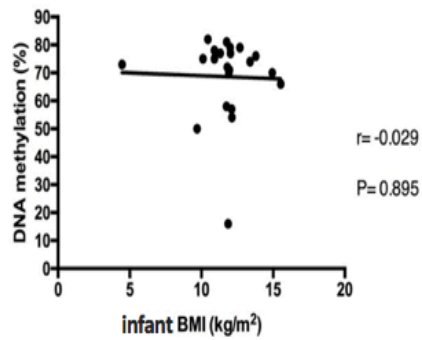


(e)



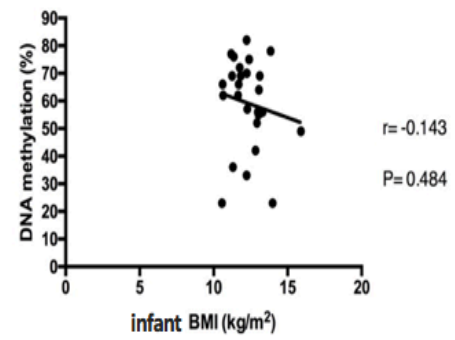
(f)

**miR-411 DNA methylation vs infant BMI in obese placenta**

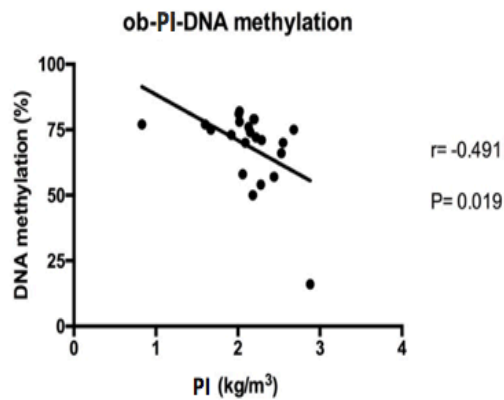


(g)

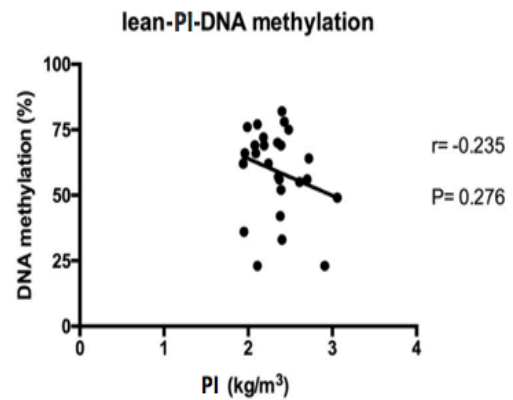
**miR-411 DNA methylation vs infant BMI in lean placenta**



(h)



(i)



(j)

**Figure 16.** Correlation between miR-411 DNA methylation and infant bw/BMI/PI/sds\_bw/sds\_BMI. (a) miR-411 DNA methylation in obese placentas vs bw. (b) miR-411 DNA methylation in lean placentas vs bw. (c) miR-411 DNA methylation in obese placentas vs sds\_BMI. (d) miR-411 DNA methylation in lean placentas vs sds\_BMI. (e) miR-411 DNA methylation in obese placentas vs sds\_bw. (f) miR-411 DNA methylation in lean placentas vs sds\_bw. (g) miR-411 DNA methylation in obese placentas vs infant BMI. (h) miR-411 DNA methylation in lean placentas vs infant BMI. (i) miR-411 DNA methylation in obese placentas vs PI. (j) miR-411 DNA methylation in lean placentas vs PI.

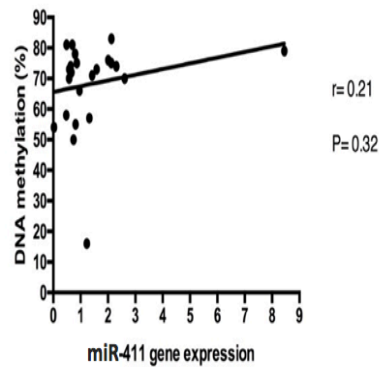
**Note:** Pearson correlation indexes “r” and “P” (significant <0.05) are shown in the figure. The straight line represents the best-fit line generated by liner regression analysis.

### **3.5.3 miR-411 DNA methylation vs gene expression in lean and obese placenta**

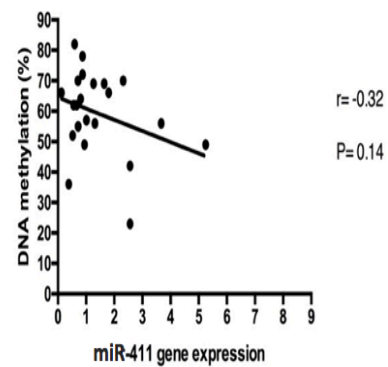
The correlation of miR-411 DNA methylation and gene expression was tested in both lean and obese placentas, but no correlation was observed in either group (Fig.17 (a,b,c,d)).

miR-411-5p gene expression vs DNA methylation in obese placenta

miR-411-5p gene expression vs DNA methylation in lean placenta



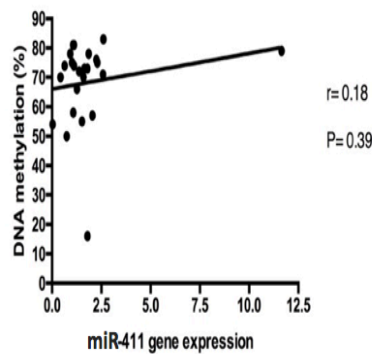
(a)



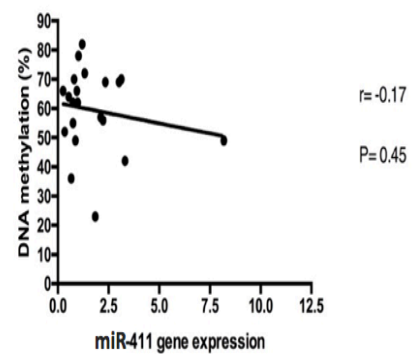
(b)

miR-411-3p gene expression vs DNA methylation in obese placenta

miR-411-3p gene expression vs DNA methylation in lean placenta



(c)



(d)

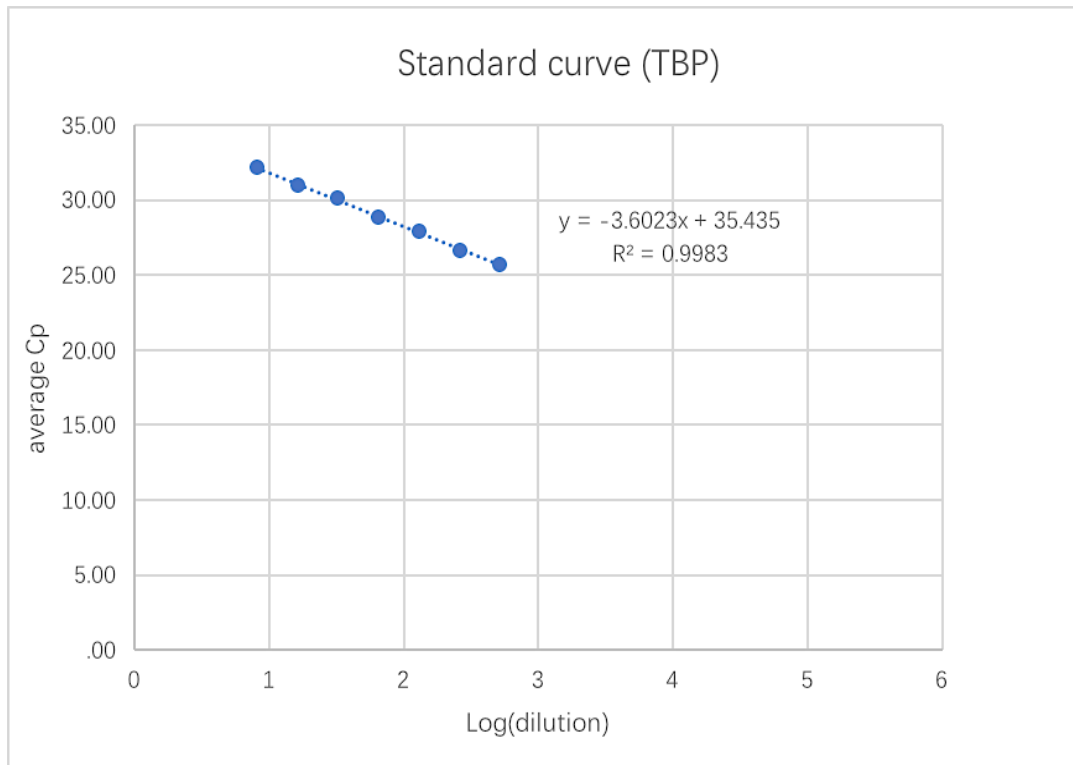
**Figure 17.** Correlation between miR-411 DNA methylation and gene expression. (a) miR-411 DNA methylation(n=25) vs miR-411-5p gene expression(n=24) in obese placentas. (b) miR-411 DNA methylation(n=22) vs miR-411-5p gene expression(n=22) in lean placentas. (c) miR-411 DNA methylation(n=25) vs miR-411-3p gene expression(n=24) in obese placentas. (d) miR-411 DNA methylation(n=22) vs miR-411-3p gene expression(n=21) in lean placentas.

**Note:** Spearman correlation indexes “r” and “P” (significant <0.05) are shown in the figure. The straight line represents the best-fit line generated by liner regression analysis.

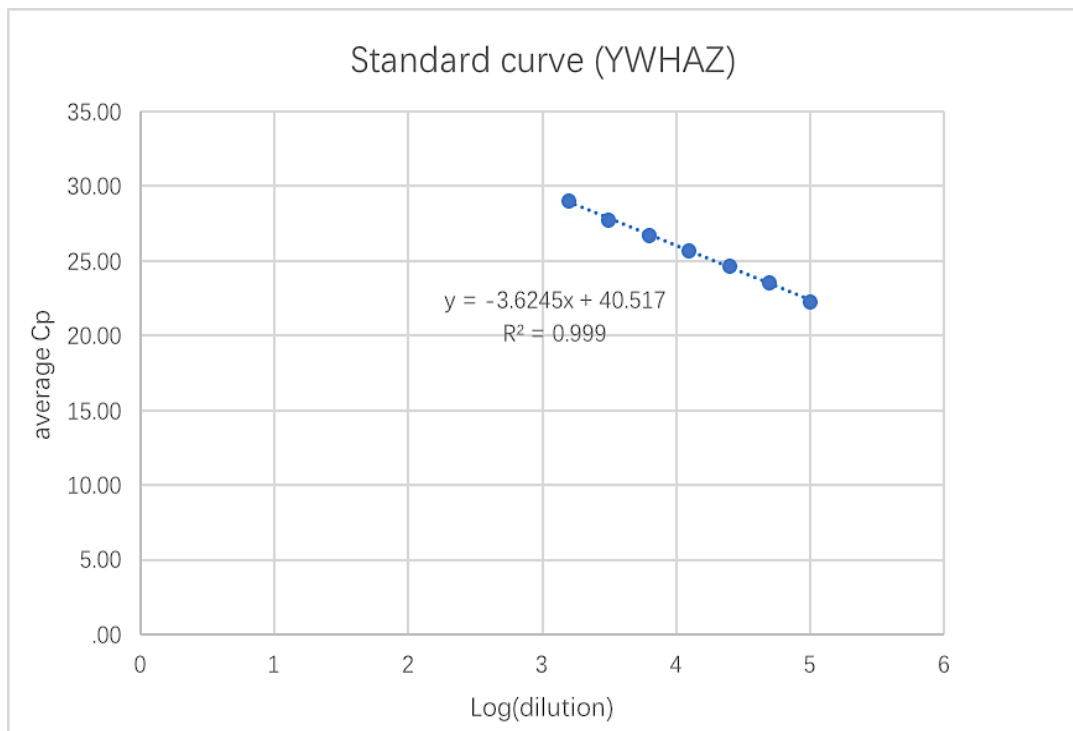


#### **3.5.4 FABP1 gene expression in lean and obese placentas**

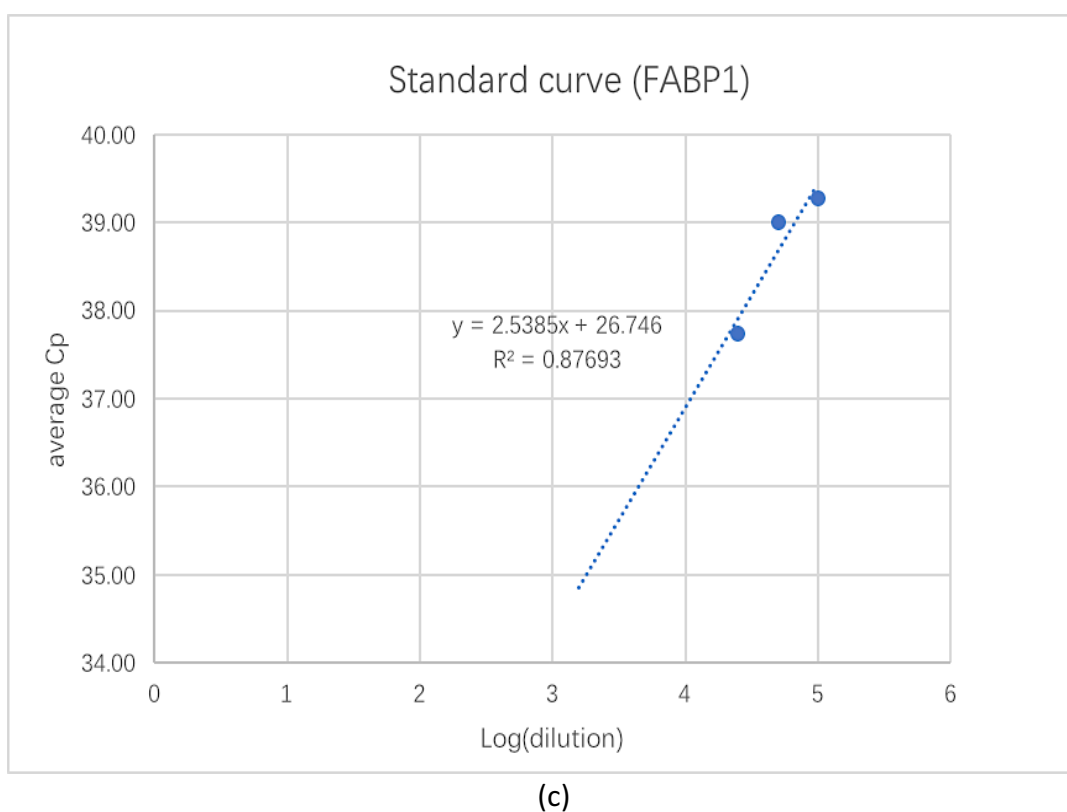
The standard curve of gene *FABP1* and housekeeping gene *TBP* and *YWHAZ* were shown in Figure 18. High efficiency PCR can be observed in housekeeping gene *TBP* and *YWHAZ* (slope<-3.32,  $R^2 \geq 0.99$ , Fig. 18(a,b)), whereas no results were shown in gene *FABP1* (Fig. 18(c)).



(a)



(b)



**Figure 18.** Standard curve of gene FABP1 and housekeeping gene TBP and YWHAZ. (a) standard curve of housekeeping gene *TBP*. (b) standard curve of housekeeping gene *YWHAZ*. (c) standard curve of gene *FABP1*. Cp means threshold cycle.

## **4 Discussion**

In our study, we demonstrated differences in DNA methylation and gene mRNA levels between placentas collected from lean and obese women. However, there were no correlations between DNA methylation and mRNA levels. It has been suggested that epigenetic mechanisms such as DNA methylation and microRNAs may underpin the DOHaD hypothesis (Wadhwa *et al.*, 2009). While investigators have started to investigate this, such as whether there is an epigenetic effect of maternal smoking on offspring brain development; and whether epigenetic gene modifications have an effect on human longevity (Toledo-Rodriguez *et al.*, 2010; Zucchi *et al.*, 2013), only few studies have been carried out to examine the influence of maternal obesity on epigenetic processes in the offspring and their later life (Swanson *et al.*, 2009; Godfrey *et al.*, 2016).

Following examination of differences between DNA methylation between placentas collected from obese and lean placentas by array, we identified three genes, miR-411, *FABP1* and *HSD17B4* for validation. These were selected as previous studies had suggested they could all be potentially relevant to fat metabolism and maternal pregnancy complications, though the function of these genes in the placenta is unknown (Atshaves *et al.*, 2010; Kozawa *et al.*, 2011; Zitman-Gal *et al.*, 2014).

The family of FABPs were demonstrated as fatty acid transporters involved in fat

metabolism in a previous study (Thumser and Storch, 2000). In our study, although we found significantly higher *FABP1* DNA methylation in obese than lean placentas, we did not detect the gene expression *FABP1* in placenta samples. The reason for this may be due to tissue specific gene expression, *FABP1* was mainly found in liver tissue (Guzmán *et al.*, 2013), and we examined placental tissue in this study.

MiR-411 mRNA levels measured in human umbilical vein cord endothelial cells have been demonstrated to decrease under diabetic conditions (Zitman-Gal *et al.*, 2014). A distinctive down-regulation of miR-411 mRNA levels was also reported in placentas collected from women with preeclampsia compared to placentas from women with uncomplicated pregnancies (Zhu *et al.*, 2009). In our study, we found higher DNA methylation in obese placenta than lean samples, but no differences of mRNA levels in those two groups. In order to understand the reason why there are no differences of miR-411 gene expression between lean and obese placentas, we further compared maternal smoking status and baby sex and infant BMI.

Smoking, one of the common adverse environmental exposures during pregnancy, has previously been shown to be associated with altered DNA methylation induced gene expression changes in whole blood samples from offspring (Markunas *et al.*, 2014; Richmond *et al.*, 2015). Smoking during pregnancy is known to have adverse

outcomes on fetal health and later life. For example, preterm birth and low birth weight are strongly associated with maternal smoking (Ng and Zelikoff, 2007). In our study, the miR-411 mRNA levels in the placentas collected from mothers in the smoking group were significantly higher than in those collected from non-smokers and ex-smokers. This result is consistent with a previous study that found 623 genes were differentially expressed between smokers and non-smokers in placental tissue (Suter *et al.*, 2011). Another study also demonstrated the influence of smoking on a specific gene *IGF2* mRNA levels (Hoyo *et al.*, 2012). However, we did not detect DNA methylation changes of miR-411. This is in accord with one previous study that mentioned 1,024 placental CpGs were found to be differently methylated between smokers and non-smokers, but only 38 CpG sites had more than 10 % differences in methylation levels (Suter *et al.*, 2011). This brings us to question the extent to which environmental exposures impact on DNA methylation alterations and whether small methylation changes can have a functional impact.

We also demonstrated highest miR-411 DNA methylation in placentas collected from male infants whereas the highest miR-411 mRNA levels were in placentas from females. While this finding needs replicating in a larger sample size, it suggests there may be sex-specific differences in DNA methylation and gene expression. Baby sex was found to be an independent factor affecting the miR-411 gene expression in lean

placentas, while maternal smoking factor was found to have an independent impact on gene expression in obese placentas. It is interesting that we found a positive correlation of infant BMI with miR-411 mRNA levels in the lean group, whereas no correlations between infant BMI and miR-411 levels were found in the obese group. This new finding suggests that miR-411 might be potentially involved in fetal fat metabolism, especially from lean pregnant women. We also found that fetal sex was independently associated with miR-411 gene expression in the lean group, and maternal smoking status was independently associated with miR-411 gene expression in the obese group, indicating the possibility that various maternal and fetal factors predict gene expressions. However, these regression models only predicted less than 50% of the variation ( $R^2 < 0.5$ ) of miR-411 gene expression by different maternal smoking status and fetal sex. As we had small sample sizes in each group in these regression models, these findings need to be replicated in a larger sample size.

Our study shows DNA methylation differences between lean and obese placentas in both *FABP1* and miR-411, but we were unable to detect differences in DNA methylation of *HSD17B4* between groups. This is the first time that DNA methylation of *HSD17B4* has been analyzed using human placenta samples. We found the DNA methylation levels of *HSD17B4* was 74.13% in obese placenta and 73.17% in lean placenta. *HSD17B4* is commonly known to be involved in the fatty



acid  $\beta$ -oxidation metabolism pathway (van Grunsven *et al.*, 1998), and mutation of this gene has been found to cause DBP deficiency (Zellweger syndrome), with symptoms including hypotonia and seizures (Suzuki *et al.*, 1997). In one human breast tumour study, DNA methylation and mRNA expression of *HSD17B4* were found to have an inverse association in human HER-2/neu-positive breast cancer using 143 tumour samples (Fiegl *et al.*, 2006). In our study we did not find differences of *HSD17B4* methylation between lean and obese placentas. The reason is unknown, but may be because different tissue types have differing DNA methylation changes or there may be differences in other DNA methylation regions that we did not study.

DNA methylation changes can be cell and tissue specific (Eckhardt *et al.*, 2006). In one study Novakovic *et al.* (2013) analyzed DNA methylation changes in the *AhRR* gene according to maternal smoking status using placenta samples, umbilical cord blood and buccal cells. They found lower *AhRR* DNA methylation level in cord blood, whereas no detectable methylation level changes in buccal cells and placenta tissues (Novakovic *et al.*, 2014). This study indicates that the influences of smoking during pregnancy can be cell and tissue specific. This is relevant to our study, as although *FABP1* is a gene mainly found in liver, due to maternal smoking exposure we could observe its DNA methylation changes in placenta tissues with no detectable

mRNA levels. As *FABP1* is a protein coding gene, it might be possible there are different protein expressions of FABP1 in lean and obese placenta samples.

In a previous study, it was demonstrated that DNA methylation could turn active genes to silenced genes (Weber *et al.*, 2007). This would mean that with higher DNA methylation levels then lower gene mRNA levels would be observed. The reason why we were unable to find any correlation of DNA methylation changes with gene expressions might due to tissue specific gene expression and maternal smoking environment. It might also be because the DNA methylation changes observed in this study were less than 10%, and how much change in DNA is needed to induce functional outcomes is still unclear. Moreover, these changes in DNA methylation and gene expressions also indicate that the placenta may play a protective role on fetal health from maternal different environment exposures. Also we would need to do functional studies to see if DNA methylation does regulate the expression of these genes (it might not).

## Strengths and limitations

The main strength of our study is the well-defined cohort with detailed information about the pregnant women and the fetus. We included placenta samples with a significantly different maternal BMI between lean and obese groups, and excluded GDM, PE and preterm birth samples. In our study, we analyzed genes and promoters potentially relevant to fat metabolism and maternal complications, and DNA methylation of *HSD17B4* was analyzed for the first time in both lean and obese placentas. We also focused on the changes in these gene and promoters with different maternal and fetal status (e.g: maternal smoking status, maternal BMI, baby sex and fetal birthweight etc.), as this has not been examined before.

One limitation in this study is that we did not have smoking samples included in lean group for miR-411 mRNA levels analysis. It would be interesting to replicate our findings in a further study with a larger number of samples to compare the changes of miR-411 mRNA levels between lean smoker and obese smoker placentas. Another limitation is that we did not have human liver samples collected in this study. It would be good to examine the mRNA levels of *FABP1* in liver tissue, to determine if the expression could be found in liver tissue but not in placentas, indicating the possibility of tissue specific gene expression. Larger sample sizes are needed to replicate the finding of fetal sex and maternal smoking status with miR-411 gene

expression and DNA methylation. Moreover, it is still not clear whether the DNA methylation changes only happening in the maternal side or the fetal side of the placenta will have the same methylation patterns and our study is limited as we had full thickness placental biopsies. Also, it is not known if the alterations in DNA methylation could be persistent throughout the adult life. Further work could focus on the epigenetic correlation between maternal and fetal and the DNA methylation changes in fetus and adulthood.

In conclusion, we observed DNA methylation changes between lean and obese placentas in both *FABPI* and miR-411 genes, and the DNA methylation level was significantly higher in obese group than lean group in both of these genes. We also demonstrated the association of maternal smoking status, baby sex and infant BMI with miR-411 mRNA levels. Further work is needed to replicate our findings and to determine whether these changes are linked with any longer term outcomes on offspring growth and development.

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